IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 595-597
Vol. 60, No. 4

IOWA STATE JOURNAL OF RESEARCH
TABLE OF CONTENTS
Volume 60
(August, 1985—May, 1986)

No. 1, August, 1985

* * * * * * * * * * *

No. 2, November, 1985

* * * * * * * * * * *

No. 3, February, 1986

* * * * * * * * * * *

No. 4, MAY, 1986

FROM THE EDITORS

The laws of physics had a peculiar fascination for me as a youngster, and I was fortunate to be allowed to pursue a course of study through my undergraduate years which confirmed and developed this interest. My first full-time job in industry was as a B.S. physicist.

The detonation of atomic bombs during the WWII years underscored how little I understood of modern physics, and I returned to graduate school, determined to understand nuclear matter and to be part of the team that would make nuclear energy beneficial to humankind. The two decades following 1950 were euphoric for nuclear physicists in terms of support and encouragement, and the fact that I did not win a Nobel prize is only evidence of my limited ability, not to the opportunitities at hand.

Now, the support has faded somewhat for nuclear physics, and another, much older field of research, is in ascendancy. Biology, the science of life, has become very exciting at its most elementary level—the molecule. Living systems, always complex, with countless dependent variables and compensating feedback networks, are being described in terms of proteins and nucleic acids. We have learned that DNA can be cut apart, modified, and reassembled; it is the template that prescribes the construction of protein molecules, which are the basic substances of living cells.

In this decade, biotechnology—the application of genetic engineering to the development of useful new products—has the excitement, support, and promise that nuclear physics had in the 1960s. The molecular biologist does not accept living forms as the finished products of evolution but is an active participant in initiating organismic change. No longer content with traditional selective breeding methods where the pace of change is tied to plant and animal life cycles, the research biologist can synthesize genes not found in nature, insert them into living cells which can reproduce, and observe the result in a laboratory environment.

As physicists did forty years ago, contemporary biologists have invaded a domain of human innocence. Can—and should—living matter be described in terms of molecules? Are not scientists who study the symmetries and beauties of nature at its most elementary level professing the most profound theism in their labors to understand the details of creation?

Daniel J. Zaffarano
Vice President for Research
Iowa State University

FOREWORD

Each year, the departments of Agronomy, Botany, Forestry, Genetics, Horticulture, and Plant Pathology, Seed and Weed Science of Iowa State University coordinate a Plant Science Lecture Series. Each series is composed of several (usually six to eight) seminars that relate to a theme. The theme is chosen to be timely and to be relevant to the genetic improvement of field, forage, horticultural, and forest crops. The theme for the 1985 Plant Science Lecture Series was "The biochemistry of host resistance to diseases and insects." This is the seventh plant science lecture series to be held on the Iowa State campus and the fifth to be published in the *Iowa State Journal of Research.*

The use of host resistance to develop crop cultivars that resist or tolerate attack from diseases and insects has been one of the most outstanding and pervasive accomplishments from plant breeding. Host resistance is effective and inexpensive to use and nonpolluting of the environment. Definitive and rapid techniques, developed to evaluate plants for reactions to pathogens and insects, have permitted the identification, selection, and large scale use of resistant cultivars. These accomplishments, spectacular as they are, have been made without knowledge of the physiological reactions involved in the pathogen-plant and insect-plant interactions. The recent knowledge and techniques available from molecular biology are beginning to provide an explanation of the biochemistry and biochemical reactions involved in plant host resistances to diseases and insects. It appears that some biochemicals that cause resistance are primary products of plant metabolism, others are secondary metabolites, and still others must be elicited by pathogen or insect attack. The papers from the 1985 Plant Science Lecture Series, published in this issue of the *Iowa State Journal of Research*, provide a review of the present knowledge about the biochemistry of host resistance to diseases and insects.

Kenneth J. Frey, Chair,
Plant Science Lecture Series Committee

IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 449-476
Vol. 60, No. 4

USE OF PHYTOTOXINS IN SELECTION OF DISEASE RESISTANT MUTANTS IN TISSUE CULTURE

Burle G. Gengenbach and Howard W. Rines[1]

ABSTRACT. Phytotoxic compounds produced by various bacterial and fungal plant pathogens have been used to select cells and plants with increased resistance to phytotoxin effects. In this report we detail the use of tissue culture selections to recover genetically defined, heritable resistance to Southern leaf blight caused by *Helminthosporium maydis* race T in corn and to Victoria leaf blight caused by *H. victoriae* in oats. Southern corn leaf blight resistance was cytoplasmically inherited and due to alterations of the mitochrondrial genome. Victoria blight resistance was a recessive trait obtained by selection in heterozygous *Vb/vb* oat tissue cultures carrying the dominant (*Vb*) allele for susceptibility. In addition, we discuss features of a model system for *in vitro* phytotoxin selection studies, briefly summarize the status of 14 other plant-pathotoxin systems described in the literature, and comment on future prospects for the use of *in vitro* phytotoxin selection in plant breeding and pathology.

Index descriptors: corn, oats, *Zea mays*, *Avena sativa*, *Helminthosporium maydis* race T, *Helminthosporium victoriae*, Southern corn leaf blight, Victoria blight, tissue culture selection, somatic cell genetics.

INTRODUCTION

The capability to initiate, maintain, and regenerate plants from tissue cultures of many agronomic and horticultural plants offers opportunities to study and modify plant disease reactions at both the cell and plant level. Many plant pathogens produce phytotoxins that are damaging to plants. The proportion of bacterial and fungal pathogens producing phytotoxins is not known, but the number recognized is increasing as additional emphasis is placed on this area of plant pathology. The number of phytotoxins that have been purified and for which chemical structure(s) have been established also is increasing. The relative importance of phytotoxins in pathogenicity and virulence and the determination of their mode of action are subjects of continuing research. Although relevant to the topic of this paper, the foregoing aspects of phytotoxins will not be dealt with in detail. These and other topics have been reviewed by Mitchell (1984), Yoder (1980), Nishimura and Kohmoto (1983), Patil (1974),

[1]Professor and Research Geneticist, USDA-ARS, respectively, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108.

Daly (1984), Daly and Deverall (1983), Strobel (1974, 1982), and Durbin (1981, 1983). Neither does the following discussion pertain to the numerous diseases in which a phytotoxin is not produced and hence cannot be used for *in vitro* selection. Rather, as indicated by the title, this specifically concerns the use of phytotoxins as *in vitro* selection agents for disease resistance.

Interest in the use of phytotoxins in selection for disease resistance was stimulated by the improvement of tobacco resistance to *Pseudomonas syringae* pv. *tabaci* as a consequence of tissue culture selection (Carlson, 1973). Carlson used methionine sulfoximine (MSO) which mimicks infection symptoms of the natural phytotoxin, tabtoxin-β-lactam, on tobacco leaves as the selective agent in tobacco protoplast and cell cultures. Both MSO and tabtoxin-β-lactam inhibit glutamine synthase activity and presumably have similar inhibitory mechanisms in tobacco cells (Thomas et al., 1983; Rennenberg and Uthemann, 1980; Leason et al., 1982). Regenerated plants from selected mutant lines expressed resistance to MSO. This resistance was controlled by one or two genes according to initial progeny tests (Carlson, 1973). Resistance to *P. syringae* pv. *tabaci* was expressed as the lack of chlorotic halos around the infection point in homozygous mutant plants. Two mutant lines had elevated free methionine in the leaf tissue, but no further studies relating to the basis of resistance have been reported.

Since 1973 there have been a number of studies in which phytoxins have been used as selective agents in plant tissue culture. We will provide detailed accounts of two studies conducted in our laboratories, discuss features of a model system for phytotoxin selection studies, and comment about the status of other plant-phytotoxin selection systems and future prospects for their use in plant breeding and pathology.

SOUTHERN CORN LEAF BLIGHT

Background

A new race of *Helminthosporium maydis* (= *Drechslera maydis*) was identified in the United States in the late 1960s. Race T differed from the previously known *H. maydis* race 0 by its specificity for corn with the Texas (T) cytoplasm source of male sterility. Fertile (N) cytoplasm and C and S sources of male sterility were not affected. Although race T and its specific damage to T-cytoplasm corn had been reported in China and the Philippines in the early 1960s, most of

the United States corn acreage was planted to hybrids with T cytoplasm. This situation led to a significant disease epidemic in 1970. National yield losses were estimated to be 15% with localized losses of 100% common (Tatum, 1971). Damage to T-cytoplasm corn was attributed to a phytotoxin (T-toxin) produced by race T but not by race O.

Miller and Koeppe (1971) demonstrated that partially purified T-toxin affected several activities of mitochondria isolated from T-cytoplasm corn. The effects of T-toxin on mitochondria may be due primarily to disruption of the inner membrane and leakage of NAD+ from the organelle (Matthews et al., 1979). Kono and Daly (1979) purified a set of β-polyketols with T-toxin specificity and later synthesized a C_{41} analogue with the same activity (Suzuki et al., 1983).

The methods for initiation, maintenance, and subsequent regeneration of plants from corn tissue cultures were being established in the early to mid 1970s (Green et al., 1974; Green and Phillips, 1975). The availability of T-toxin preparations that elicited cytoplasm-specific effects and a developing corn tissue culture system provided an excellent opportunity to combine them into an *in vitro* selection project.

Establishment of Cuture Selection Conditions

Initial tests of T-toxin were conducted with nonregenerable tissue cultures initiated as previously described (Green et al., 1974; Gengenbach and Green, 1975). The T-toxin preparation was partially purified by ethyl acetate extraction of race T *H. maydis* culture filtrates and was relatively impure. Completely purified toxin was not required, because resistant control cultures of N-cytoplasm corn were used to test for nonspecific effects of T-toxin preparations. However, the *H. maydis* liquid culture media had to be modified to eliminate inhibition of N tissue cultures by the T-toxin preparations. Yeast extract and ammonium tartrate were eliminated from the fungal culture medium, 2% glucose replaced 3% sucrose, and the mineral salts were reduced 90% compared to the original Fries No. 3 medium. T-toxin preparations used for selection were not toxic to N cultures but effectively inhibited growth of T-cytoplasm tissue cultures (Table 1).

Selection of T-toxin resistance in tissue cultures of corn inbred A619T (T-cytoplasm) resulted in the isolation of many resistant cell lines. These lines were stable over time without continued selection pressure and had mitochondria that were no longer sensitive to T-toxin (Gengenbach and Green, 1975).

Table 1. Effect of partially purified T-toxin preparation[a] on growth of A619N and A619T corn tissue cultures.[b]

Culture	T-toxin concentration in medium (%)				
	0	.1	.5	1	5
	% of control				
A619N	100	88	98	83	105
A619T	100	57	34	17	0

[a]Preparation was concentrated 20-fold from original culture filtrate.

[b]Reference: Gengenbach and Green (1975).

Selection and Regeneration of Resistant Plants

Results with the A619T cultures indicated selection for resistance was feasible, but these cultures were not amenable to regeneration of plants. Green and Phillips (1975) in the meantime developed the procedures for establishment of corn tissue cultures capable of plant regeneration. A genotype which worked especially well for regeneration was the inbred A188. Crosses were made to produce a genotype which had T-cytoplasm and 75% of the A188 nuclear genes. Embryos of this genetic constitution were isolated at an immature stage (1-2 mm long, 12 days postpollination). Cultures were initiated and increased through subculture on control media for about six months (Gengenbach et al., 1977).

These cultures were subjected to a stepwise selection for T-toxin resistance. The initial T-toxin level was relatively non-inhibitory, and the cultures were selected visually for the fastest growth and for retention of the culture appearance that was associated with plant regeneration capability. At succeeding subculture steps, the T-toxin concentration either remained the same or was increased depending on the severity of inhibition and the amount of viable callus remaining. Because this was the first experience with selection in regenerable corn cultures, it was important to try to insure that resistant cell lines also would be capable of plant regeneration.

The first nine plants obtained following the fourth subculture selection cycle (six months of selection) all gave susceptible reactions when leaves were treated with T-toxin. These plants were cytoplasmically male sterile, and their progeny bred true for susceptibility

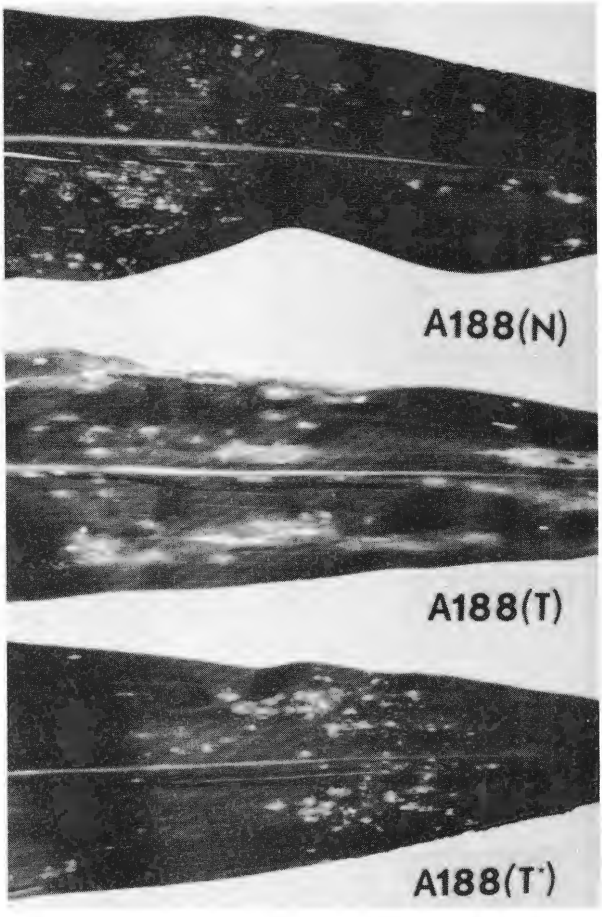


Figure 1. Lesion development of *Helminthosporium maydis* race T on leaves of field grown plants of resistant control (A188N), susceptible control (A188T), and a resistant line (A188T*) derived from tissue culture selection for T-phytotoxin resistance.

and sterility. Plants regenerated after the fifth and subsequent subculture selection cycles were resistant to the T-toxin, and a majority also were fully male fertile. Selection continued for a period of about 18 months; however, only resistant tissue culture lines remained after about ten months. A total of 167 resistant plants was regenerated from at least 18 independently derived resistant tissue culture lines (Gengenbach et al., 1981). Ninety-seven of these plants were male fertile. The remaining plants failed to shed pollen due to abnormal morphology and probably to cytological and genetic aberrations.

Evaluation of Disease Resistance

Progeny of resistant and susceptible plants regenerated from tissue culture were inoculated with spores of *H. maydis* race T under greenhouse conditions favoring disease development. After ten days, the lesion size on the five-week-old seedling leaves was measured. Lesion sizes on the selected resistant progeny were the same as on control N cytoplasm plants. Lesions on the susceptible T-cytoplasm control plants and on susceptible regenerated progeny were significantly larger.

Additional tests were made with an advanced generation of a resistant selected line in the field. An inoculum of infected leaf material which had been ground was applied into the whorl of plants at the 7-8 leaf stage. Although lesion measurements were not taken, observations throughout the growing season indicated that the selected resistant line and the N-cytoplasm control were equivalent in field resistance (Figure 1). Secondary spread of the disease on T-cytoplasm control plants resulted in extensive leaf death by the time of grain filling. Genotypes of *H. maydis* that do not produce T-toxin cause small lesions on resistant as well as susceptible corn (Yoder, 1980). T-toxin, therefore, is considered to be a factor in virulence and to alter the degree of damage or severity of disease. Selection for T-toxin resistance in tissue culture resulted in resistant plants that are totally insensitive to T-toxin effects as a virulence factor, but toxin insensitivity did not confer total resistance to race T infection.

Inheritance of Resistance and Male Fertility

As indicated earlier, regenerated plants exhibited three phenotypes: resistant and male fertile, susceptible and male sterile like the initial cultured material, and resistant but male sterile. Resistant male

fertile regenerated plants were either self-pollinated or pollinated with A188N males. They also were crossed onto T-cytoplasm females. Susceptible, male sterile regenerated plants were pollinated by A188N males. These produced only susceptible, male sterile progeny. Resistant, male sterile regenerants were pollinated with A188N, but no seed were produced. This lack of seed set suggested that a high degree of female sterility or other physiological aberrations were associated with the male sterility of these selected resistant plants and that the basis of their sterility was not likely due to T-cytoplasm.

Inheritance data were obtained for resistant, male fertile plants during two or three generations of selfing or backcrossing (Gengenbach et al., 1981). T-toxin resistance was transmitted only through the female resistant parent and not through the male (Table 2). Toxin resistance was clearly under extra nuclear or cytoplasmic genetic control.

Inheritance of male fertility also was cytoplasmically controlled, but progeny test data differed slightly from the toxin test data. In the first generation after selfing or backcrossing the resistant, male fertile regenerants, several families segregated male sterile plants; however, in subsequent generations no male sterile plants were obtained (Table 3). Progeny obtained by crossing the first generation male sterile plants with A188N males were all male fertile indicating that sterility was not due to the cytoplasmic condition. Rather, it was probably due to recessive nuclear mutations or to chromosomal alterations. Male fertility was not transmitted in crosses of T-cytoplasm females with the selected resistant fertile males. This result shows that male fertility in the selected regenerants was not due to nuclear male fertility restorer genes.

Succeeding backcross generations of ten resistant, male fertile selected lines have now been grown in the field at St. Paul, Minnesota, for eight years. When tested for T-toxin resistance, these advanced generations have been resistant, and all plants have been fully male fertile. These traits have shown no instability.

Other Altered Traits

T-cytoplasm corn is susceptible to *Phyllosticta maydis* and to a phytotoxin produced by the fungus (Comstock et al., 1973). Resistant and susceptible lines from tissue culture were tested for reaction to *P. maydis* phytotoxin preparations. In all cases, plants resistant to T-toxin also were resistant to *P. maydis* phytotoxin (Gengenbach et al., 1981). Susceptible plants were susceptible to both toxins. Recently

Table 2. Inheritance of resistance to *Helminthosporium maydis* T-toxin in progeny of regenerated resistant male-fertile plants (R).[a]

Source of progeny[b]	Number of families	Number of plants	
		Resistant	Susceptible
R selfed	68	1054	0
R backcrossed	27	579	0
T × R crosses	68	0	3000

[a]Data from Gengenbach et al., 1981.

[b]Includes two selfed generations, three generations of T × R crosses using Wf9T and A619T × A188 females as T parents and R lines as recurrent males.

Danko et al. (1984) have shown that the two toxins have similarities in chemical structure and therefore might be expected to have similar specificity even though produced by different pathogenic fungi. The insecticide methomyl also specifically affects T-cytoplasm corn at the whole plant and mitochondrial level (Koeppe et al., 1978). Preliminary tests of selected resistant seedlings indicated they were not inhibited by methomyl (Chourey and Gengenbach, unpublished data). These

Table 3. Inheritance of male fertility in progeny of regenerated resistant male-fertile plants (R).[a]

Source of progeny[b]	Number of families	Number of plants	
		Male fertile	Male sterile
R selfed once	8	152	43
R backcrossed once	20	290	5
R selfed twice	7	97	0
R backcrossed 2 or 3 times	8	397	0
T × R crosses	94	0	7424

[a]Data from Gengenbach et al., 1981.

[b]See Table 2 for descriptions; includes three generations of T × R crosses.

concomitant changes in *P. maydis* and methomyl resistance and in male fertility brought about by selection *in vitro* for T-toxin resistance suggest that there is a common biochemical and/or genetic basis.

Molecular Analysis of Mitochrondrial DNA

The cytoplasmic inheritance of these multiple traits indicated control either by the chloroplast or mitochondrial genomes. Restriction endonuclease digestion and electrophoresis of chloroplast DNA (ctDNA) and mitochondria DNA (mtDNA) has been used to differentiate plant cytoplasm sources (Sederoff, 1984). The mtDNA from ten independently selected, resistant male-fertile lines and T- and N-cytoplasm controls were compared by restriction analysis. All ten resistant lines showed one common mtDNA fragment alteration when compared to the parental T-cytoplasm control following digestion by the enzyme Xho I or Pst I (Gengenbach et al., 1981; Hartloff and Gengenbach, unpublished). These mtDNA changes are illustrated in Figure 2. Although mtDNA changes are known to arise as a consequence of regeneration of plants from T-cytoplasm corn tissue cultures (Gengenbach and Pring, 1982), the changes shown in Figure 2 have only been associated with the change from a susceptible and male sterile phenotype to a resistant and male fertile phenotype. Susceptible, male sterile lines derived from T-cytoplasm corn tissue cultures have not had the specific alterations shown in Figure 2 (Gengenbach, unpublished). Thus, the region of the T-cytoplasm mitochondrial genome defined by the 6.6 kb Xho fragment and 10 kb Pst fragment may contain or be near the gene or genes for southern leaf blight susceptibility and male sterility. The loss of a T-cytoplasm-specific, 13 kd mitochondrial protein also occurs in lines with the altered 6.6 kb Xho and 10 kb Pst mtDNA fragments (Dixon et al., 1982; Gengenbach, unpublished).

Somaclonal Variation for Resistance

T-toxin resistant, male fertile plants have been obtained from T-cytoplasm corn tissue cultures even though the cultures were not subjected to selection for toxin resistance (Brettell et al., 1980; Umbeck and Gengenbach, 1983; Hartloff and Gengenbach, unpublished). The frequency of such plants varies but is much less than for cultures selected for T-toxin resistance. Changes in mtDNA and in the 13 kd mitochondrial protein also are like those in the selected lines suggesting a common genetic mechanism for selected and unselected

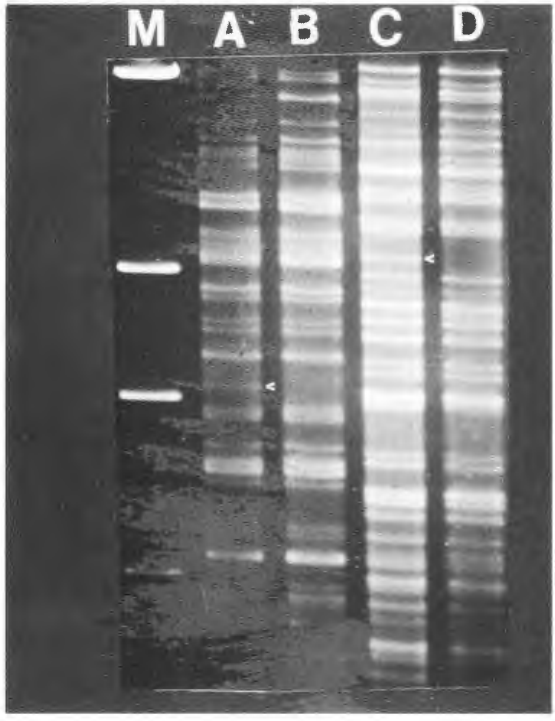


Figure 2. Electrophoretic patterns of mitochondrial DNA from parental susceptible, male-sterile A188T (lanes A, C) and a selected resistant, male-fertile line (lanes B, D) following digestion by Xho I (lanes A, B) and Pst I (lanes C, D) restriction endonucleases. Lane M contains lambda DNA size markers of 23.5, 9.6, 6.6, and 4.5 kb, respectively, from the top. The 6.6 kb Xho I fragment and the 10.0 kb Pst I fragment characteristic of T-cytoplasm but altered in resistant fertile lines are indicated by arrows between lanes A and B and lanes C and D, respectively.

T-toxin resistant lines. Selection for toxin resistance in culture probably is only hastening the somatic assortment of cells already altered or capable of mtDNA alterations responsible for resistance.

VICTORIA BLIGHT OF OATS

Background

Victoria blight (incited by *H. victoriae* Meehan and Murphy = *Bipolaris victoriae* Shoem.) became epidemic in oats due to widespread use of genetic lines selected for a specific purpose but uniquely susceptible to Victoria blight. The oat cultivar 'Victoria' was introduced into United States oat breeding programs in 1927 because of its resistance to races of crown rust (*Puccinia coronata* Cda. f. sp. *avenae* Fraser and E. Led.), which had periodically devastated United States oat crops up to that time. Only after numerous cultivars containing the major gene for crown rust resistance from Victoria were occupying the majority of the oat acreage of the United States was it realized that this gene coincidentally imparted susceptibility to Victoria blight (Meehan and Murphy, 1946). This realization and the rapid spread of Victoria blight caused a replacement of oat cultivars with ones having new sources of crown rust resistance. These lines were not susceptible to Victoria blight or to the highly specific toxin (victorin) produced by it.

The Victoria blight susceptibility (*Vb*) locus in oats has many properties which make it a highly interesting model system for studying selection for phytotoxin resistance in tissue culture. Susceptibility to Victoria blight and resistance to oat crown rust are inherited together as a simple nuclear dominant trait. Oats lacking the *Vb* allele are resistant to 100,000 times the level of *H. victoriae* toxin that produces 50% inhibition in root growth bioassays in susceptible oats (Luke and Wheeler, 1955). Sensitivity to victorin toxin and susceptibility to the *H. victoriae* pathogen itself have shown complete correlation between resistant and susceptible oat lines and among progeny from crosses between them (Litzenberger, 1949). Victorin has been highly purified (Walton and Earle, 1984) although its structure and mode of action are as yet unknown. Victorin appears to affect cell membranes because its presence is associated with a rapid loss of electrolytes from cells of susceptible lines (Wheeler and Black, 1962). A common mechanism has been postulated for the victorin necrotic response that allows invasion by the facultative saprophyte, *H. victoriae*, and the hypersensitive crown rust reaction that stops the spread of the obligate parasite, crown rust (Meehan and Murphy, 1947; Litzenberger, 1949). Several hundred victorin-resistant mutants have been isolated from radiation and chemically mutagenized *Vb Vb* oat lines by selecting segregating seedlings for ability to grow in the

presence of the phytotoxin (Wheeler and Luke, 1955; Wallace and Luke, 1961). In all cases, the victorin resistant selections also lost their original resistance to crown rust. The high specificity of the phytotoxin, the known nuclear inheritance pattern of resistance, and the coincident trait of crown rust resistance make the victorin system in oats an extremely interesting model system both for developing methodology for efficient selection systems in cereal tissue cultures and for analyzing the type of mutants that may be produced in *in vitro* cultures.

Rationale for Tissue Culture Selection

Because resistance to victorin is a recessive trait, the direct recovery of resistant lines from a homozygous sensitive *Vb Vb* oat culture would require two mutational events. The probability of this occurring would be low and recovery of resistance would require a high level of selection. Such a situation might well be encountered in other selection schemes for phytotoxin resistance and was considered to be worth investigating. Haploid *Vb* oat lines could be used, but recovery of haploid oat plants from anther culture is rare (Rines, 1983). No stable regenerable haploid tissue cultures have been reported in any cereal. The alternative approach followed was to make the selections in cultures that had been initated from heterozygous *Vb vb* toxin susceptible oat lines. These were produced as the F_1's from crosses of homozygous susceptible *Vb Vb* lines by homozygous resistant *vb vb* lines.

Components of the Selection System

The phytotoxin used in the selection medium was highly purified victorin prepared by H. H. Luke, USDA-ARS plant pathologist at Gainesville, Florida (Rines and Luke, 1985). The victorin had been concentrated and purified from an *H. victoriae* culture filtrate by a series of washes with organic solvents and passages through an acid alumina column and a Bio-Gel P-4 column (exclusion limits 3,600 daltons). The final product, when lyophilized, was stable to long term storage at -15°C and provided a consistent source of toxin of known concentration for selection studies. The lyophilized material produced 50% root growth inhibition at 10^{-7} dilution in standard bioassays (Luke and Wheeler, 1955). Phytotoxin diluted to 5×10^{-5} was chosen for selection because it was a "marginally lethal" level in preliminary tests of susceptible tissue on a dilution series of toxin.

The *Vb Vb* oat lines used to initiate tissue cultures were the cultivars 'Victoria' and 'Victorgrain.' Heterozygous *Vb vb* culture lines were initiated from F_1 embryos produced by crosses of Victoria by the *vb vb* line 'GAF.' GAF is an oat line developed specifically for its high frequency tissue culturability and regeneration capacity (Rines and Luke, 1985). Tissue cultures were initiated from immature embryos, and plants were regenerated as had been described previously (Rines and McCoy, 1981).

Selection and Regeneration of Resistant Plants

When homozygous *Vb Vb* culture lines consisting of six callus pieces each were placed on toxin selection medium, all callus growth ceased, and there were no surviving tissues after about 45 days (Table 4). In contrast, there were surviving tissues in 16 of 175 callus pieces representing 13 of 22 heterozygous *Vb vb* culture lines placed on toxin-containing medium (Table 4). These surviving tissues ranged from large, vigorously growing callus sectors to single shoots arising from a callus piece. When surviving tissues were transferred to regeneration medium, plants were obtained for 11 of the 13 culture lines. These plants were then tested for their sensitivity to victorin in a leaf bioassay, in which the cut end of a leaf was placed in a toxin solution. Sensitive leaves had a marked wilting response after two to three days while leaves from resistant controls remained turgid. Regenerated plants from nine of the eleven regenerable toxin-selected cultures were insensitive to the toxin. The two sensitive plants probably were escapes from toxin selection in culture, because the only surviving tissue had been small shoots. Vigorous callus was obtained in most of the other cultures from which insensitive plants were regenerated. It was observed that during the tissue culture selection on toxin-containing medium, the more differentiated regions in a heterogeneous culture tended to survive longer. Apparently, uptake or diffusion of toxin into these tissues was slower than in less differentiated tissue. This tendency for the more differentiated tissues to survive longer on selection medium has been noted by the authors in selections using other growth inhibiting chemicals. In contrast to the *H. maydis* system in corn described earlier, there were no *H. victoriae* toxin-insensitive plants among regenerants of *Vb vb* oat tissue cultures which had never been exposed to toxin containing medium (Table 4).

Table 4. Tissue culture initiation, selection for toxin resistance, and plant regeneration in homozygous *Vb Vb* and heterozygous *Vb vb* victorin-sensitive oats.[a]

	Vb Vb	*Vb vb*	
	Toxin selection	Toxin selection	No toxin selection
No. embryos placed onto culture initiation medium	86	38	46
No. embryo-derived culture lines (total no. callus pieces) placed on toxin-containing medium	14 (84)	22 (175)	---
No. embryo-derived culture lines (total no. callus pieces) with surviving tissue after 45 to 60 days on toxin medium	0	13 (16)	---
No. embryo-derived culture lines with regenerated plants and their toxin reactions in leaf bioassays	0	9 Insensitive 2 Sensitive	0 Insensitive 30 Sensitive

[a]Data from Rines and Luke, 1985.

Evaluation of Disease Resistance in Progeny

Plants from five of the nine culture lines with toxin-insensitive regenerants set seed as did plants of the two lines with toxin-sensitive regenerants. Lack of seed set in regenerated plants is not uncommon due either to genetic abnormalities or to residual physiological effects from the tissue culture process. All progeny of toxin-insensitive regenerants were toxin-insensitive, thus demonstrating that the tissue culture selected toxin insensitivity was a genetically stable change. Progeny of the two toxin-sensitive regenerants segregated three sensitive: one insensitive just as the original *Vb vb* toxin-sensitive line put into culture. This result further indicates that the two toxin-sensitive regenerants had simply been escapes from selection.

The same progeny plants were also tested for their reactions to race 202 of crown rust. Progeny of victorin toxin-insensitive regenerants were 100% susceptible in the crown rust test, while progeny of victorin-sensitive regenerants segregated three resistant: one susceptible to crown rust. Thus, in these tissue culture selections as in other mutagenic and recombination experiments, loss of sensitivity to *H. victoriae* toxin was accompanied by loss of resistance to crown rust. Toxin-insensitive plants recovered following selection in tissue culture were not tested for their reactions to *H. victoriae* but were assumed to be resistant, since earlier work had shown toxin susceptibility to be essential to *H. victoriae* pathogenesis (Litzenberger, 1949).

Origin of Toxin-Insensitive Variants in Culture

One possible origin of loss of victorin sensitivity in the *Vv vb* oat lines taken through tissue culture selection would be loss of the chromosome or chromosomal segment with the *Vb* locus. A high frequency of chromosome breakage and partial chromosome loss observed in plants regenerated from oat tissue cultures (McCoy et al., 1982) makes this situation highly possible. However, no major chromosomal deficiency was detected in cytological analysis of meiotic tissues of the regenerated toxin-insensitive plants. A simple point mutation or small deletion in the *Vb* locus is another possible origin of the selected toxin insensitivity. An intermediate level of toxin insensitivity, as was observed in certain mutants recovered by conventional mutagenesis and toxin screening of seedlings, would be indicative of a point mutation. None of the toxin-insensitive tissue culture selections described here were of an intermediate type. Somatic recombination in cultures, as apparently occurs in *Su su* tobacco cells in culture

(Lorz and Scowcroft, 1983), could also produce a *vb vb* toxin-insensitive culture sector. The occurrence of such a somatic recombination event is not detectable without flanking markers or a means to detect a reciprocal *Vb Vb* product. However, it remains a likely origin of insensitive selections.

The extremely high selectivity of the *H. victoriae* toxin in sensitive oats make this an attractive system for testing effects of treatments with chemical mutagens, variation in culture conditions, and other factors on the frequency of genetic changes and efficiency of tissue culture selection schemes in plant tissue cultures.

CONSIDERATIONS FOR PHYTOTOXIN SELECTIONS IN TISSUE CULTURE

Model System

At least four features seem important to us in establishing a model or optimal system for using phytotoxin selection in plant tissue culture. The following discussion does not imply an order of importance because in some situations one can compensate for another.

1. Purified phytotoxin with established chemical structure.
2. Mode of phytotoxin action determined at cellular and whole plant levels.
3. Plant tissue cultures from which plant regeneration is readily obtained.
4. Plant genotypes with different expression of resistance which can be tested in tissue culture.

1. **Purified phytotoxin with established chemical structure.** Purification of a single compound or set of related compounds with phytotoxic activity is desirable, because the concentration of the phytotoxin can then be established. Selection conditions can be defined accurately and repeatably from one phytotoxin preparation to another. This would reduce the chance of tissue culture selection media being prepared with an ineffective or a completely lethal phytotoxin level. An acceptable alternative, if the phytotoxin is stable in storage, is to prepare a large batch so that selections and tests can be made using a common lot with predictable concentration effects. Availability of purified phytotoxin is not an absolute requirement because it may not be feasible to obtain sufficient quantities for large scale *in vitro* selections, and partially purified phytotoxins have been used successfully. There is a need, however, to determine that the

inhibitory effect of a phytotoxin preparation is due to the phytotoxin it contains rather than to other inhibitory compounds that are not related to the phytotoxin action or disease susceptibility. Lack of pure phytotoxin can be alleviated by testing the phytotoxin preparation on tissue cultures of plant genotypes known to be resistant to the phytotoxin and/or the disease. An inhibitory response in such tests could be attributed to general inhibitors in the preparation or to the possibility that plant resistance was caused by factors not functioning at the culture level. In the latter case, additional resistance might still be selectable in culture. Knowledge of the phytotoxin chemical structure could provide information about stability and solubility in tissue culture media, uptake into tissue culture cells, and perhaps possible cellular target sites if that was not already known.

2. Mode of phytotoxin action determined at cellular and whole plant levels. Knowledge of the mode of action of a phytotoxin at the whole plant and cellular or subcellular levels can provide the rationale or justification for initiating tissue culture selection. For example, it was known that T-toxin affected the activity of isolated mitochondria as well as mitochondria in intact tissues of T-cytoplasm corn. Although the primary site of action was not determined until later and the exact mechanism of damage is still not clear, this information stimulated interest in selection at the time corn tissue culture methods were developing. The mode of action is established for only a few phytotoxins (e.g., tentoxin, tabtoxin-β-lactam and phaseolotoxin; Mitchell, 1984), and even fewer have been confirmed in plant tissue culture cells.

Mode of action information does not guarantee, however, that selection in tissue culture will be successful. The primary mechanism of toxin action in whole plants may not be expressed in tissue culture cells; for example, the phytotoxin may inhibit an enzyme required in phototrophic cells, but this enzyme is not active or its activity is not necessary for survival of cells in culture conditions. Conversely, and perhaps less likely, is the situation in which other cellular activities are inhibited by a phytotoxin in tissue culture cells but not in vivo. Selection for phytotoxin resistant tissue cultures may not result in phytotoxin resistant plants in this case. In addition, there is the possibility that the type of change required to prevent phytotoxin interaction and cellular damage also is deleterious to normal cellular functions or even lethal. While mode of action information is useful in planning selection of phytotoxin resistant tissue cultures, it might not predict the outcome and does not substitute for the actual experiment.

3. Plant tissue cultures from which plant regeneration is readily available. For applied tissue culture selection work, plant regeneration is required. The sophistication of the tissue culture selection procedure and the method of plant regeneration are of secondary importance as long as plants can be obtained in some fashion following selection. Protoplast systems have been used for few phytotoxin selection experiments (Carlson, 1973). For most studies to date, suspension cell cultures and tissue cultures on solid media have been used (Tables 5—8). Protoplast systems could provide a means to determine the frequency of phytotoxin resistant cells in a large population, if this information is needed for better understanding of the host-pathogen relationship. Several studies also have been conducted in which somaclonal variation for disease resistance has been evaluated following regeneration from protoplasts of potato (Matern et al., 1978; Shepard et al., 1980). Reliable protoplast culture and plant regeneration methods are not currently established for grass and cereal plant species. For these plants, callus or suspension culture methods continue to be useful. Haploid cultures would be preferred if the phytotoxin resistance is a recessively inherited trait. Also, if resistance is multigenic, faster progress could be expected in a haploid culture.

4. Plant genotypes with different expression of resistance which can be tested in tissue culture. The availability of host genotypes with differential reaction to a disease organism or to races of a pathogen have been invaluable in host-pathogen studies. Genotypic differences can be very useful in establishing the appropriate tissue culture conditions for phytotoxin selection. Tissue cultures of a genotype with whole plant resistance could be established to test whether resistance to the phytotoxin also is expressed at the cellular level. Such expression would indicate that selection within tissue cultures of susceptible genotypes may result in resistant plants. Tissue cultures of resistant genotypes also can be used to determine whether the phytotoxin preparation has deleterious effects on culture growth that are caused by constituents other than the phytotoxin. This is especially useful when the phytotoxin preparation has not been or can not be purified completely.

CURRENT STATUS AND FUTURE PROSPECTS

At least 16 studies involving 15 different plant tissue culture-phytotoxin systems have been reported to date (Tables 5—8). This

Table 5. Selection of genetically defined disease resistance using phytotoxins in tissue culture.

Plant	Disease organism	Genetic control	Reference
Tobacco	*Pseudomonas syringae* pv. *tabaci*	1 Mutant with 2 recessive genes 2 Mutants with semidominant gene	Carlson, 1973
Corn	*Helminthosporium maydis*	Cytoplasmic, mitochondrial	Gengenbach et al., 1977 Brettell et al., 1980
Oats	*Helminthosporium victoriae*	Recessive gene	Rines and Luke, 1985

Table 6. Selection of heritable disease resistance using phytotoxins in tissue culture.

Plant	Disease organism	Progeny tests	Reference
Tobacco	*Pseudomonas syringae* pv. *tabaci*	26% progeny resistant	Thanutong et al., 1983
Tobacco	*Alternaria alternata*	9% progeny resistant	Thanutong et al., 1983
Rape	*Phoma lingam*	50-75% tolerant progeny from tolerant plants	Sacristan, 1982
Sugarcane	*Helminthosporium sacchari*	73% stably resistant in 5 vegetative generations	Larkin and Scowcroft, 1983

Table 7. Disease resistance expressed in regenerated plants following phytotoxin selection in tissue culture.

Plant	Disease organism	Characteristics	Reference
Potato	*Phytophthora infestans*	25% reduction in lesion size	Behnke, 1980a
Potato	*Fusarium oxysporum*	Increased resistance in plants, possible chimeric origin	Behnke, 1980b
Alfalfa	*Fusarium oxysporum*	Plants resistant to pathogen New callus resistant to phytotoxin	Hartman et al., 1984

Table 8. Preliminary results from use of phytotoxins in tissue culture.

Plant	Disease organism	Observations	Reference
Wheat	*Pseudomonas syringae* pv. *syringae*	Callus growth inhibited by syringomycin	Pauly, 1983
Wheat	*Septoria nodorum*	Plants regenerated after 2 culture selection cycles	Brettell[a], 1984
Rice	*Helminthosporium oryzae*	Callus response to toxin corresponds to genetic resistance or susceptibility to the fungus	Vidhyusekaran[b], 1984
Geranium	*Xanthomonas campestris* pv. *pelargonii*	Callus growth inhibited	Hammerschlag et al., 1982
Pearl millet	*Claviceps fusiformis*	Callus growth inhibited	Bajaj et al., 1980
Tomato	*Stemphylium botryosum* pv. *lycopersici*	Suspension cells inhibited	Barash et al., 1982

[a]Brettell, R. I. S. 1984. Personal communication.

[b]Vidhyusekaran, P. 1984. Personal communication.

represents a small portion of the potential plant-phytotoxin combinations that could be tested for selection in tissue culture. Most of the studies have been reported in the last five years, and some of them are currently in progress. The results to date seem quite encouraging although complete information is not yet available for most systems. The information needed for application purposes includes: (1) expression of phytotoxin resistance in regenerated plants, (2) mode of inheritance of phytotoxin resistance, and (3) the degree of disease resistance afforded by expression of phytotoxin resistance.

Nine of the studies have shown that regenerated plants were resistant or at least less susceptible to the phytotoxin and/or to the pathogen. Resistance was shown to be transmitted to progeny in seven of these studies, but the inheritance pattern has been established in only three studies to date. In the corn and oat examples, the inheritance data fit the same pattern as for the available resistant genotypes. Additional genetic information undoubtedly will become available as other work progresses.

Selection for phytotoxin resistance in plant tissue cultures has resulted in improved disease resistance in the regenerated plants and/or their progeny. Progeny homozygous for resistance to MSO did not produce the chlorotic halo typical of *P. syringae* pv. *tabaci* inoculation or MSO treatment (Carlson, 1973). Similar results were obtained for tobacco cultures selected for resistance to *P. syringae* pv. *tabaci* phytotoxin (Thanutong et al., 1983). Tobacco cultures selected for resistance to *Alternaria alternata* phytotoxin also produced plants with significantly reduced density of necrotic flecks on leaves inoculated with the pathogen (Thanutong et al., 1983). Behnke (1980a) reported a 25% reduction in lesion size after inoculating regenerated potato plants with *Phytophthora infestans*. These plants were regenerated from cultures selected for survival on culture filtrates of *P. infestans*. Whether this type of change would be significant in disease resistance was not reported. Selection for *Phoma lingam* phytotoxin resistance in rape tissue cultures resulted in plants with only small necrotic spots or with greatly reduced symptoms after stem inoculations (Sacristan, 1982). Alfalfa plants regenerated after selection for resistance to *Fusarium oxysporum* phytotoxin gave disease ratings for inoculated roots ranging from zero to three compared to four for the parental line and 1.9 for a resistant genotype (Hartman et al., 1984). All but two regenerated plants had ratings less than or equal to the resistant control.

In addition to inheritance and disease tests, culture-derived resistant genotypes must be evaluated for other performance

characteristics relative to the parental or source genotype. None of the examples listed in Tables 5—8 have led to this type of agronomic evaluation yet. These tests may be performed without changing the genetic background of the selected plants. In most plant breeding programs, evaluations also would need to be done after incorporating the selected trait into other elite genotypes. As many independently derived resistant plants as possible should be obtained during the tissue culture selection process to improve the likelihood that some will survive the scrutiny of disease and agronomic performance evaluations.

The number of crop plants that can be regenerated from tissue culture is still increasing and now includes nearly all major crop species. Work on phytotoxin purification, identification, mode of action, and importance in disease also continues to expand. Taken together these two trends should indicate additional opportunities to select for phytotoxin resistance in plant tissue cultures. Whether it occurs will depend partly on existing types and sources of resistance, the ease with which resistance could be incorporated into the germplasm of interest, and access to the tissue culture and phytotoxin technology.

The most benefit may be derived in situations where there is little or no genetic resistance or where resistance is available only in inferior germplasm. If tissue culture regeneration procedures are in place, selection for phytotoxin resistance may be the most practical means to obtain genetic material for improving disease resistance. The four attributes of a model system as discussed previously will likely not be achieved, but the selection should be attempted. It may be relatively straightforward to select for "resistant" tissue cultures, regenerate plants, and test them for altered reaction to the disease organism.

Selection of tissue cultures and plants resistant to a phytotoxin can help establish the importance of the phytotoxin in pathogenicity and virulence. Resistance to the pathogen may be partially accomplished by whole plant mechanisms that do not include cellular resistance to a phytotoxin. Combining the two types of resistance into one genotype may result in an improved level of resistance not achieved by either alone.

LITERATURE CITED

Bajaj, Y. P. S., P. S. Phul, and S. K. Sharma. 1980. Differential tolerance of tissue cultures of pearl-millet to ergot-extract. Indian J. Exp. Biol. 18:429-432.

Barash. I., G. Pupkin, D. Netzer, and Y. Kashman. 1982. A novel enolic β-ketoaldehyde phytotoxin produced by *Stemphylium botryosum* f. sp. *lycopersici*. Plant Physiol. 69:23-27.

Behnke, M. 1980a. General resistance to late blight of *Solanum tuberosum* plants regenerated from callus resistant to culture filtrates of *Phytophthora infestans*. Theor. Appl. Genet. 56:151-152.

————. 1980b. Selection of dihaploid potato callus for resistance to the culture filtrate of *Fusarium oxysporum*. Z. Pflanzenzucht. 85:254-258.

Brettell, R. I. S., E. Thomas, and D. S. Ingram. 1980. Reversion of Texas male-sterile cytoplasm maize in culture to give fertile, T-toxin resistant plants. Theor. Appl. Genet. 58:55-58.

Carlson, P. S. 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366-1368.

Comstock, J. C., C. A. Martinson, and B. G. Gengenbach. 1973. Host specificity of a toxin from *Phyllosticta maydis* for Texas cytoplasmically male-sterile maize. Phytopathology 63:1357-1360.

Daly, J. M. 1984. The role of recognition in plant disease. Ann. Rev. Phytopathology 22:273-307.

———— and B. J. Deverall. 1983. Toxins and Plant Pathogenesis. Academic Press, New York.

Danko, S. J. and collaborators. 1984. Structure and biological activity of a host-specific toxin produced by the fungal corn pathogen *Phyllosticta maydis*. Biochemistry 23:759-766.

Dixon, L. K., C. J. Leaver, R. I. S. Brettell, and B. G. Gengenbach. 1982. Mitochondrial sensitivity to *Drechslera maydis* T-toxin and the synthesis of a variant mitochondrial polypeptide in plants derived from maize tissue cultures with Texas male-sterile cytoplasm. Theor. Appl. Genet. 63:75-80.

Durbin, R. D. 1981. Toxins in Plant Disease. Academic Press, New York.

————. 1983. The biochemistry of fungal and bacterial toxins and their modes of action, pp. 137-162. *In*: J. A. Callow (ed.). Biochemical Plant Pathology. J. Wiley, New York.

Gengenbach, B. G. and C. E. Green. 1975. Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin. Crop Sci. 15:645-649.

————, C. E. Green, and C. M. Donovan. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Natl. Acad. Sci. USA 74:5113-5117.

———— and D. R. Pring. 1982. Isolation of revertants from CMS-T by tissue culture techniques, pp. 257-261. *In*: W. F. Sheridan (ed.). Maize for Biological Research, Plant Molecular Biology Association, Charlottesville, VA.

————, J. A. Connelly, D. R. Pring, and M. F. Conde. 1981. Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. Theor. Appl. Genet. 50:161-167.

Green, C. E. and R. L. Phillips. 1975. Plant regeneration from tissue cultures of maize. Crop Sci. 15:417-421.

————, ————, and R. A. Kleese. 1974. Tissue cultures of maize (*Zea mays* L.): initiation, maintenance, and organic growth factors. Crop Sci. 14:54-58.

Hammerschlag, F. A., P. J. Bottino, and R. N. Stewart. 1982. Effect of culture filtrates of *Xanthomonas campestris* pv. *pelargonii* and extracts of stems inoculated with *X.* c. pv. *pelargonii* on geranium callus and seedlings. Plant Cell Tissue Organ Cult. 1:247-254.

Hartman, C. L., T. J. McCoy, and T. R. Knous. 1984. Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxin(s) produced by *Fusarium oxysporum* f. sp. *medicaginis* Plant Sci. Lett. 34:183-194.

Koeppe, D. E., J. K. Cox, and C. P. Malone. 1978. Mitochondrial heredity: A determinant in the toxic response of maize to the insecticide methomyl. Science 201:1227-1229.

Kono, Y. and J. M. Daly. 1979. Characterization of the host-specific pathotoxin produced by *Helminthosporium maydis*, race T, affecting corn with Texas male sterile cytoplasm. Bioorg. Chem. 8:391-397.

Larkin, P. J. and W. R. Scowcroft. 1983. Somaclonal variation and eyespot toxin tolerance in sugarcane. Plant Cell Tissue Organ Cult. 2:111-121.

Leason, M. and collaborators. 1982. Inhibition of pea leaf glutamine synthetase by methionine sulphoximine, phosphinothricin and other glutamate analogues. Phytochemistry 21:855-857.

Litzenberger, C. S. 1949. Inheritance of resistance to specific races of crown and stem rust, to *Helminthosporium* blight, and of certain agronomic characters in oats. Iowa State Agr. Expt. Stn. Res. Bull. 370.

Lorz, H. and W. R. Scowcroft. 1983. Variability among plants and their progeny regenerated from protoplasts of *Su/su* heterozygotes of *Nicotiana tabacum*. Theor. Appl. Genet. 66:67-75.

Luke, H. H. and H. E. Wheeler. 1955. Toxin production by *Helminthosporium victoriae*. Phytopathology 45:453-458.

Matern, U., G. Strobel, and J. Shepard. 1978. Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc. Natl. Acad. Sci. USA 75:4935-4939.

Matthews, D. E., P. Gregory, and V. E. Gracen 1979. *Helminthosporium maydis* race T toxin induces leakage of NAD+ from T cytoplasm corn mitochondria. Plant Physiol. 63:1149-1153.

McCoy, T. J., R. L. Phillips, and H. W. Rines. 1982. Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue cultures: High frequency of partial chromosome loss. Can. J. Genet. Cytol. 24:37-50.

Meehan, F. and H. C. Murphy. 1946. A new *Helminthosporium* blight of oats. Science 104:413-414.

———— and ————. 1947. Differential phytotoxicity of metabolic by-products of *Helminthosporium victoriae.* Science 106:270-271.

Miller, R. J. and D. E. Koepe. 1971. Southern corn leaf blight: susceptible and resistant mitochondria. Science 173:67-69.

Mitchell, R. E. 1984. The relevance of non-host-specific toxins in the expression of virulence by pathogens. Ann. Rev. Phytopathol. 22:215-245.

Nishimura, S. and K. Kohmoto. 1983. Host-specific toxins and chemical structures from *Alternaria* species. Ann. Rev Phytopathol. 21:87-116.

Patil, S. S. 1974. Toxins produced by phytopathogenic bacteria. Ann. Rev. Phytopathol. 12:259-279.

Pauly, M. H. 1983. Evaluation of a selection system for *Pseudomonas syringae* pv. *syringae* pathotoxin resistance in wheat tissue culture. M. S. Thesis, University of Minnesota, St. Paul, MN. 77 pp.

Rennenberg, H. and R. Uthemann. 1980. Effects of L-methionine-S-sulfoximine on growth and glutathione synthesis in tobacco suspension cultures. Z. Naturforsch. 35:945-951.

Rines, H. W. 1983. Oat anther culture: Genotype effects on callus initiation and the production of a haploid plant. Crop Sci. 23:268-272.

———— and H. H. Luke. 1985. Selection and regeneration of toxin-insensitive plants from tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae.* Theor. Appl. Genet. 71:16-21.

———— and T. J. McCoy. 1981. Culture initiation and plant regeneration in hexaploid species of oats. Crop Sci. 21:837-842.

Sacristan, M. D. 1982. Resistance responses to *Phoma lingam* of plants regenerated from selected cell and embryogenic cultures of haploid *Brassica napus.* Theor. Appl. Genet. 61:193-200.

Sederoff, R. R. 1984. Structural variation in mitochondrial DNA. Adv. Genet. 22:1-108.

Shepard, J. F., D. Bidney, and E. Shahin. 1980. Potato protoplasts in crop improvement. Science 208:17-24.

Strobel, G. A. 1982. Phytotoxins. Ann. Rev. Biochem. 51:309-333.

————. 1974. Phytotoxins produced by plant parasites. Ann. Rev. Plant Physiol. 25:541-566.

Suzuki, Y. and collaborators. 1983. Comparison of activities of the host-specific toxin of *Helminthosporium maydis,* race T, and a synthetic C_{41} analog. Plant Physiol. 73:440-444.

Tatum, L. A. 1971. The Southern corn leaf blight epidemic. Science 171:1113-1116.

Thanutong, P., I. Furusawa, and M. Yamamoto. 1983. Resistant tobacco plants from protoplast-derived calluses selected for their resistance to *Pseudomonas* and *Alternaria* toxins. Theor. Appl. Genet. 66:209-215.

Thomas, M. D., P. J. Langston-Unkefer, T. F. Uchytil, and R. D. Durbin. 1983. Inhibition of glutamine synthetase from pea by tabtoxinine-β-lactam. Plant Physiol. 71:912-915.

Umbeck, P. F. and B. G. Gengenbach. 1983. Reversion of male-sterile T-cytoplasm maize to male fertility in tissue culture. Crop Sci. 23:584-588.

Walton, J. D., and E. D. Earle. 1984. Characterization of the host-specific phytotoxin victorin by high-pressure liquid chromatography. Plant Sci. Lett. 34:231-238.

Wallace, A. T. and H. H. Luke. 1961. Induced mutation rates with gamma rays at a specific locus in oats. Crop Sci. 1:93-96.

Wheeler, H. E. and H. S. Black. 1962. Changes in permeability induced by victorin. Science 137:983-984.

———— and H. H. Luke. 1955. Mass screening for disease resistant mutants in oats. Science 122:1229.

Yoder, O. C. 1980. Toxins in pathogenesis. Ann. Rev. Phytopathol. 18:103-129.

IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 477-499
Vol. 60, No. 4

PHYTOALEXINS AND THEIR INVOLVEMENT IN PLANT DISEASE RESISTANCE

N. T. Keen[1]

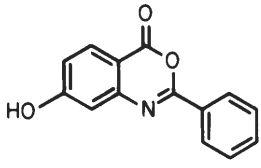
ABSTRACT. Phytoalexins are antibiotic secondary metabolites produced by plants in response to certain pathogens and appear to be involved in disease resistance. While different plants utilize various pathways to biosynthesize phytoalexins of several different structures, they all share the properties of inducible production and antibiosis. Students of phytoalexins have made considerable progress in the last few years in defining the biochemical and molecular changes in plant metabolism that contribute to phytoalexin production as well as the role of pathogen-produced metabolites, called elicitors, in initiation of their production. This paper reviews the role of phytoalexins in disease resistance and recent progress in understanding their biosynthetic regulation.

Index descriptors: elicitors, disease resistance genes, phytoalexins, hypersensitive response, Ti vectors, specific resistance, general resistance.

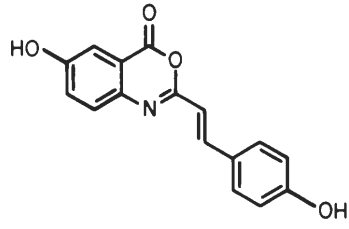
INTRODUCTION

Accelerated investigation of naturally occurring disease resistance reactions in higher plants has taken place the last 15 years. Much of this research has been directed to the study of inducibly-produced antibiotic chemicals called phytoalexins. While they have not been shown to occur in all plants, an imposing body of evidence supports the basic proposition that phytoalexin production constitutes a widespread and important defense mechanism against pest attack. I will review some of this evidence as well as discuss recent progress in understanding the biosynthesis of phytoalexins. Finally, I will present some speculations on how this information may permit the development of novel disease control measures. The paper will concentrate only on recent research, since several reviews have appeared during the last few years (Bailey and Mansfield, 1982; Darvill and Albersheim, 1984; Grisebach, 1983; Grisebach et al., 1982; Mansfield, 1983; Smith, 1980).

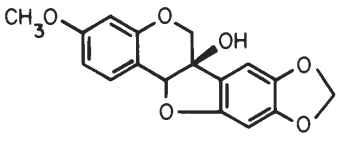
[1]Department of Plant Pathology, University of California, Riverside, CA 92521.



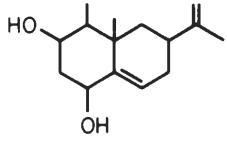
DIANTHALEXINE
FROM CARNATION



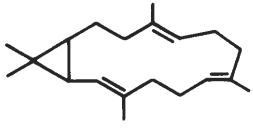
AVENALUMIN I
FROM OAT



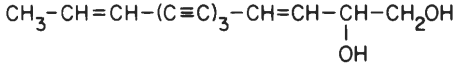
PISATIN
FROM PEA



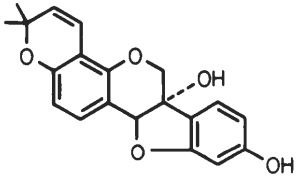
CAPSIDIOL
FROM PEPPER



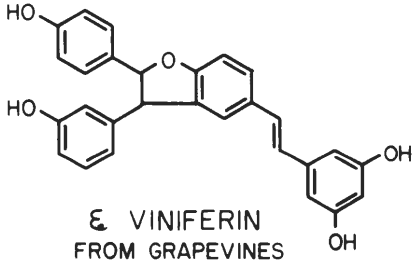
CASBENE
FROM CASTOR BEAN



SAFYNOL
FROM SAFFLOWER



GLYCEOLLIN I
FROM SOYBEAN



ε VINIFERIN
FROM GRAPEVINES

Figure 1. Structures of some representative phytoalexins.

THE NATURE OF PHYTOALEXINS

Phytoalexins are low molecular weight antibiotic products of secondary plant metabolism that are inducibly formed after attack by a pest or pathogen. Recently isolated phytoalexins have often been found to possess previously undescribed chemical structures. Some representative phytoalexins are shown in Figure 1. The avenalumins from oats (Mayama et al., 1981) and dianthalexine from carnation (Bouillant et al., 1983) are the only nitrogen-containing phytoalexins thus far discovered. Otherwise, several sesquiterpenoid phytoalexins of the Solanaceae such as capsidiol (Kuć, 1982), many of the legume isoflavonoid phytoalexins such as pisatin and glyceollin (Ingham, 1982), stilbenes such as the viniferins (Langcake and Pryce, 1977), safynol (Allen and Thomas, 1971), casbene (Sitton and West, 1975) and others are novel compounds. Despite the fact that various plants make different phytoalexin structures, there, nevertheless, are definite structure-activity requirements for phytoalexin activity. This is shown by the fact that small structural alterations frequently result in greatly decreased antibiotic activity (Keen, 1981). Coupled with the general uniqueness of phytoalexin structures, this suggests that higher plants have evolved the biochemical pathways needed for phytoalexin production.

Although new phytoalexins are described regularly, little effort has been made to assess the variety of phytoalexins made across a broad spectrum of higher plants. A notable exception is the extensive systematic research of John Ingham and his colleagues with legume phytoalexins (see Ingham, 1982 for a review of much of this work). In addition to the description of several previously unknown phytoalexins, Ingham's work has shown that the phytoalexin structures produced by a certain plant species may have considerable chemosystematic value. This was confirmed by Keen et al. (1985), who compared the phytoalexins produced by various accessions of the genus *Glycine*. Seven different but closely related glyceollins were observed among 61 accessions of ten *Glycine* species, but considerable qualitative and quantitative variation occurred in the phytoalexins produced by different accessions. Some currently defined species showed similar patterns of glyceollin production among accessions collected from several geographic locations, indicating that they are in fact closely related. Other species, however, exhibited considerable variation in the glyceollins produced by accessions. It is possible these findings have taxonomic significance.

Phytoalexins were first studied in connection with fungal diseases of plants, but initially there was uncertainty concerning their antibiotic properties to other pathogens and pests such as bacteria and insects. Recent work has shown that, while differences in sensitivity certainly exist, some bacteria are as sensitive to the inhibitory effects of phytoalexins as fungi (Lyon, 1984; Pankhurst and Biggs, 1980; Weinstein and Albersheim, 1983). Surprisingly, we have little data concering the effects of phytoalexins on the development and behavior of insects and even less on their role, if any, in effecting resistance to insect pests of plants. There are a few reports that demonstrate that phytoalexins are deleterious to insects and arachnids and may markedly influence insect feeding patterns (Biggs et al., 1983; Hart et al., 1983; Russell et al., 1978; Sutherland et al., 1980).

Recent work has extended earlier research (VanEtten and Pueppke, 1976) on the mechanism of toxicity of phytoalexins. For instance, Weinstein and Albersheim (1983) have shown that the soybean isoflavonoids glycinol, glyceollin, and coumestrol inhibited the structural integrity of membranes in Gram-negative bacteria. While glyceollin also exhibits relatively specific inhibitory effects on electron transport in isolated soybean mitochondria (Boydston et al, 1983; Kaplan et al., 1980), the results support the idea that phytoalexins function as non-specific membrane antagonists and do not have discrete target sites. Indeed, with the exception of pathogens such as those described in the next section that have evolved specialized mechanisms permitting tolerance to or degradation of host phytoalexins (VanEtten and Stein, 1978; Smith et al., 1984), their toxicity appears similar in all cells.

PHYTOALEXINS AS MEDIATORS OF DISEASE RESISTANCE

Phytoalexin production in intact plant tissues is often associated with a necrosis of host cells surrounding the pathogen infection site, called the hypersensitive response (HR). In this paper, resistance implies the capacity of a plant to restrict pathogen multiplication and/or colonization to a level less than that occurring in a susceptible plant. A resistant plant reaction denotes an incompatible plant-pathogen interaction while a susceptible reaction denotes a compatible interaction. The phytoalexin/HR mechanism is involved in at least two types of resistance. One type, called *general*, is effective against a wide range of microorganisms and against all biotypes of a certain pathogen species. The second type of disease resistance, called *specific*, is controlled by single genetic alleles, referred to as disease

resistance genes. General resistance is believed to be a major contributor to the fact that most potential pathogens are unsuccessful on any single plant. On the other hand, single disease resistance genes have been successfully used for some 80 years as the major control strategy for many diseases important in agriculture. Perhaps because disease resistance genes are usually effective against only a single pathogen species, certain biotypes of the pathogen may evolve which "overcome" the resistance. These "virulent races" frequently do not elicit plant phytoalexin production as did their avirulent ancestors. The relationship between resistance genes in host plants and virulence genes in pathogens is currently an actively investigated area (see Keen, 1985).

Following the discovery of phytoalexins, there was considerable reluctance by many scientists to accept their role in naturally occurring disease resistance. Consequently, a great deal of effort has been expended in testing this association. While phytoalexins certainly do not account for all cases of disease resistance, an imposing body of evidence links phytoalexins with the expression of resistance in certain cases (Table 1). One example of a previous uncertainty is the interaction of *Aphanomyces euteiches* with its host, the pea plant. VanEtten and Pueppke (1976) found that the fungus was highly sensitive to the antibiotic effects of pisatin *in vitro* but observed that the fungus rapidly colonized pea plants despite the fact that the infected tissue accumulated high concentrations of the phytoalexin. This apparent contradiction of the phytoalexin concept was employed to seriously question the general role of phytoalexins in disease resistance (Pueppke, 1978). Recently, Sweigard and VanEtten (1984) have found at least a partial explanation of the *A. euteiches* anomaly. They demonstrated that highly polar lipids extracted from pea plants decreased the *in vitro* sensitivity of the fungus to pisatin when they were incorporated into culture media. The same compounds did not affect the sensitivity of other pathogenic fungi to the phytoalexin. It is accordingly likely that growth of *A. euteiches* but not the other fungi in pea plants containing pisatin is due to the protective effect of the pea polar lipids. Thus, instead of experimentally detracting from a defensive role for phytoalexins, the *Aphanomyces*-pea interaction is an interesting case in which the fungus may escape effects of the pisatin defense system similar to certain other successful pathogens of plants and animals (see Keen, 1981).

A common test of phytoalexin involvement in disease resistance is whether the phytoalexin is produced at the pathogen infection site in sufficient quantity to account for cessation of pathogen growth (Table 1).

Table 1. Summarized data indicating that phytoalexins constitute a basis for disease resistance in plants. References are not exhaustive, but generally are to recent papers or review articles.

1.	Compatible pathogens often chemically degrade or are more tolerant to the phytoalexins produced by their host plant than are other organisms (Smith et al., 1984; Tegtmeier and VanEtten, 1982).
2.	Pretreatment of normally susceptible plants with phytoalexin elicitors may result in their subsequent resistance (Darvill and Albersheim, 1984; Keen, 1981).
3.	General and specific inhibitors or environmental alterations that block phytoalexins production also decrease the expression of disease resistance (Mayama et al., 1982a; Moesta and Grisebach, 1982; Yoshikawa et al., 1978a).
4.	In gene-for-gene systems, high phytoalexin production is only observed in incompatible plant-pathogen combinations but not in compatible interactions (Keen, 1982; Mayama et al., 1982b).
5.	The speed of phytoalexin production in resistant reactions of varying efficiency is positively correlated with the degree of pathogen inhibition (Long et al., 1985; Mayama et al., 1982a).
6.	The localized concentration of phytoalexins at the growing margin of an incompatible pathogen is sufficiently high to account for growth inhibition (Hahn et al., 1985; Moesta et al., 1982).
7.	Coinoculation of mixed compatible and incompatible pathogen races results in restricted development of both, and high phytoalexin levels are produced (see Keen, 1981; Long et al., 1985).
8.	Pathogen elicitors and suppressors reproduce the same relative specificity for phytoalexin production as the living pathogen (DeWit et al., 1985; Keen, 1985; Tietjen and Matern, 1984).

While phytoalexins have been assumed to exhibit equal activity in the plant as *in vitro*, recent work has suggested that several phytoalexins are in fact more active *in vivo* (Bakker et al., 1983; Essenberg, personal communication). Enhanced toxicity in these cases was found to result from conversion of the phytoalexins to free radicals as a result of photooxidation. Such a mechanism may clearly be of considerable significance in field situations.

Much experimentation has shown that the host cell necrosis associated with the HR is not causally related to restricted pathogen development (Harder et al., 1979; Mayama et al., 1982a). However, it is unclear whether hypersensitive necrosis is a requirement for subsequent phytoalexin accumulation or whether the necrosis occurs coincidentally with or is caused by the events which initiate phytoalexin production. As I will discuss later, considerable evidence indicates that the primary mechanism accounting for phytoalexin accumulation in plant tissues is the derepressed transcription and translation of several genes coding for biosynthetic enzymes. Considerable evidence, however, indicates that necrotic, hypersensitive plant cells function as a reservoir for the accumulation of phytoalexins synthesized and exported from surrounding healthy cells (Nakajima et al., 1975; Sakai et al., 1982). Since the dead plant cells appear to have reduced activities of enzymes that degrade the phytoalexins, this may be important in accounting for the high local concentrations of phytoalexin which accumulate at infection sites of incompatible pathogens.

Two particular host-pathogen systems have supplied evidence supporting the functional involvement of phytoalexins in plant disease resistance. The first is the pea-*Fusarium solani* f. sp. *pisi* system. It illustrates a case of general resistance and has been utilized in incisive genetic experiments by VanEtten and his colleagues. Various isolates of the fungus exhibited differing pathogenicity, and this correlated with their ability to detoxify chemically the pea phytoalexin, pisatin, by demethylation. To test critically the association between pathogenicity, tolerance to pisatin, and pisatin demethylase activity, genetic crosses were performed between highly pathogenic, pisatin tolerant isolates and pisatin sensitive isolates of low pathogenicity (Kistler and VanEtten, 1984; Tegtmeier and VanEtten, 1982). Insensitivity to pisatin, the production of inducible pisatin demthylase activity and high pathogenicity were linked in all cross progeny tested. The experiments, therefore, provide convincing evidence that (1) high pathogenicity in *F. solani* f. sp. *pisi* requires pisatin demethylase activity; and (2) pisatin production may confer

resistance in pea plants against isolates of the fungus that do not produce the demethylase activity. Thus, these genetic experiments provide persuasive evidence in support of the role of pisatin as a resistance factor in peas.

The second system which has been investigated in depth is the soybean-*Phytophthora megasperma* f. sp. *glycinea* system. This interaction exemplifies specific resistance and behaves as a gene-for-gene system. Earlier work with the system has been reviewed by Grisebach et al. (1982) and Keen and Bruegger (1977) and will not be reiterated here. Several recent papers by Ward and colleagues (e.g., Lazarovits et al., 1981; Ward and Lazarovits, 1982) have questioned the role of glyceollin in resistance expression. However, etiolated soybean seedlings were employed, and efforts were not made to quantitate localized glyceollin levels in the inoculated hypocotyls. Since the expression of disease resistance is a multicellular plant response of closely packed cells (Holliday and Klarman, 1979), it is difficult to see the resemblance of etiolated soybean plants to the normal, pigmented plants occurring in nature. Finally, several of the experiments by Ward's group employed alterations in environment after inoculation that resulted in a change in plant disease reactions. For example, Ward and Lazarovits (1982) showed that fungus growth terminated concomitant with accumulation of high concentrations of glyceollin in and around the infection site of an incompatible fungus race when inoculated plants were maintained at 22°C. When the temperature was raised to 30°C, the fungus resumed colonization of the plant, but total glyceollin remained high. Since the high phytoalexin levels formed at 22°C were assumed to prevent subsequent fungus growth, the authors questioned the role of glyceollin in resistance expression. Such an argument neglects the possibility that one or more of the hundreds of hyphal tips in the infection site might escape the glyceollin barrier following the shift to 30°C. The occurrence of such escapes at 22°C would not be of consequence since they would initiate additional glyceollin production in the newly invaded cells. Because soybean cells surrounding the original infection site did not produce more phytoalexin at 30°C, however, any fungal hyphae which escaped the pre-existing barrier at this temperature would grow unchecked, and the plant would become susceptible. Unfortunately, Ward and Lazarovits did not test this possibility by studying localized glyceollin accumulation in areas occupied by hyphal tips.

Investigators who have employed techniques to determine localized glyceollin levels have consistently concluded that the concentration formed in resistant-reacting plants at the margin of fungal colonies

is indeed sufficient to account for cessation of fungus development (Grisebach et al., 1982; Hahn et al., 1985; Keen et al., 1982; Moesta et al., 1983; Yoshikawa et al., 1978b). Grisebach's group has recently developed two elegant approaches to the study of phytoalexin localization in inoculated plants. In the first, laser microprobe mass analysis (Grisebach et al., 1982; Moesta et al., 1982) was employed to demonstrate that glyceollin in fact accumulated in a narrow zone surrounding the infection site of an incompatible *P. megasperma* f. sp. *glycinea* race. Inoculation with a compatible race resulted in lower local concentrations of the phytoalexin that occurred, however, over a larger volume of host tissue. Moesta et al. (1983) developed a radioimmune assay (RIA) for glyceollin I, the major isomer formed by soybean hypocotyls and roots. This technique was also employed by Hahn et al. (1985) to demonstrate that glyceollin is produced more rapidly in plants inoculated with an incompatible race of *P. megasperma* f. sp. *glycinea* than with a compatible race. Further, the sensitive RIA assay detected glyceollin at only two hours after inoculation. Initial phytoalexin production thus occurred several hours earlier than detected previously by less sensitive techniques. This discovery raises the question of the importance of hypersensitive host cell death as a prerequisite to phytoalexin production. None of the many groups that have studied the soybean-*P. megasperma* f. sp. *glycinea* system have yet conducted a careful study of the timing of hypersensitive host cell death during the expression of resistance.

Another recent line of experimentation which strongly supports the role of glyceollin in resistance to *P. megasperma* f. sp. *glycinea* is the use of relatively specific inhibitors of glyceollin formation. Plant protein synthesis inhibitors such as blasticidin S (Yoshikawa et al., 1978a) have previously been shown to block glyceollin production and resistance expression, but it was possible that these inhibitors also blocked other active defense mechanisms. Aminooxyphenylpropionic acid and glyphosate are more specific inhibitors of the shikimic acid pathway in soybean and have been shown to block both glyceollin production and resistance to incompatible *P. megasperma* f. sp. *glycinea* races (Keen et al., 1982; Moesta and Grisebach, 1982). The experiments therefore support involvement of the phytoalexin with disease resistance.

The two host-pathogen systems above have supplied much of the evidence indicating a functional role for phytoalexins in disease resistance, but several others have yielded data consistent with phytoalexin involvement. We can conclude that the HR/phytoalexin mechanism is likely an important resistance mechanism.

PHYTOALEXIN ELICITORS

I have recently reviewed this rapidly moving area (Keen, 1985) and will accordingly only summarily discuss it here. It has been known for some time that various fractions, called elicitors, can be isolated from plant pathogens that will initiate phytoalexin production in the appropriate plant tissue. Unfortunately, several different elicitors have often been recovered from the same pathogen, and this raises the question of which, if any, are physiologically involved in the interactions occurring between pathogen and host plant. Exciting recent progress has occurred with specific resistance via the isolation of elicitors that possess race specific properties (Anderson, 1980; De Wit et al., 1985; Mayama and Keen, 1984). These elicitors are produced by pathogen races that are incompatible on the tester plant resistance genotype but not by corresponding compatible races of the pathogen. The elicitors are efficient in host cultivars that are resistant to the pathogen race in question but not on susceptible plant cultivars. Unlike pathogen elicitors that show no race-cultivar specificity (Hadwiger and Bechman, 1980; Sharp et al., 1984), their differential activity argues that the race-specific elicitors are in fact physiologically important in plant-pathogen interactions.

The occurrence of race/cultivar specific elicitors does not necessarily imply that non-specific pathogen elicitors are without function. It is suspected that widely conserved components such as fungal cell wall glucans and chitin, bacterial outer membrane elements, and nematode cell surface glycoproteins may elicit general resistance in non-host plant species (Keen, 1982). Successful pathogens would, according to this hypothesis, have evolved means for preventing recognition of their non-specific elicitors by host plants or would have evolved mechanisms for tolerance or detoxification of host phytoalexins.

The recent molecular cloning of avirulence genes from two bacterial pathogens (Gabriel, 1984; Staskawicz et al., 1984) is expected to result in definitive attempts to determine if their primary gene products are race specific elicitors. The work to date has demonstrated that the various avirulence genes express the incompatible phenotype when transferred to other bacterial races. The gene cloning work is accordingly consistent with the occurrence in these systems of an elicitor-receptor model, to be discussed in the next section.

HOW PHYTOALEXIN ELICITORS FUNCTION

The available evidence is most consistent with a model in which pathogen elicitors bind to plant receptors, presumably on the plasma membrane (Keen, 1982; Yoshikawa, 1983). The elicitor-receptor complexes then lead, by an unknown mechanism, to derepression of certain plant genes involved in phytoalexin biosynthesis. There is substantial experimental support for the induction of certain phytoalexin biosynthetic enzymes after elicitor treatment, but relatively little evidence is available on the occurrence of plant receptors for elicitors and none on the nature of the postulated intermediates that function between elicitor binding and DNA transcription.

Yoshikawa et al. (1983) provided the first experimental evidence that receptors which specifically bind the glucan elicitor mycolaminaran may occur on soybean cells. However, since this elicitor does not possess race specific properties, the physiological relevance of the receptors is unclear. A more convincing case for occurrence of a putative receptor which recognizes a race-specific phytoalexin elicitor emanates from the recent demonstration by Mayama and Keen (1984) that the host-specific toxin, victorin, is an efficient phytoalexin elicitor in *Pc-2* oat cells but not in those of the victorin insensitive *pc-2* genotype. The *Pc-2* allele is interesting because it appears to confer both sensitivity to victorin and resistance to certain races of the crown rust fungus, *Puccinia coronata* f. sp. *avenae*. Thus *Pc-2* is a classical single gene for disease resistance. Mayama et al. (1982b) have shown that resistance conferred by *Pc-2* and several other crown rust resistance genes is due to production of the avenalumins (Fig. 1). Further, extensive work by Scheffer and colleagues (for review see Scheffer and Livingston, 1984) previously suggested that victorin functions by binding to a specific receptor protein occurring only on the plasma membrane of *Pc-2* oat cells. The hypothesis therefore is that victorin mimics a race-specific elicitor produced by *P. coronata* f. sp. *avenae* and binds to the *Pc-2* encoded receptor to elicit phytoalexin production. The correctness of this model should be testable through attempts to isolate the victorin receptor.

We do not know whether the nuclear transcription involved in photoalexin production results from direct migration of the elicitor-receptor complex to the plant nucleus or whether second messengers mediate this effect. Suggestions that ethylene may be involved as an elicitor or second messenger have been disproven in several plants (Bufler and Bangerath, 1982; Chappell et al., 1984; Paradies et al., 1980). Hahn and Grisebach (1983) also have obtained evidence

indicating that cAMP does not play this role. It has been suggested that "endogenous" or "constitutive" plant elicitors (Bailey, 1982; Hargreaves, 1981; Moesta and Grisebach, 1981), perhaps pectic oligosaccharides (Bruce and West, 1982; Davis et al., 1984), may function as second messengers, especially in response to abiotic elicitors that damage plant cells. Dixon et al. (1983) observed that green bean cells exposed to an abiotic elicitor, but not a fungal elicitor, released a dialyzable factor(s) which functioned as an endogenous elicitor. This may indicate that endogeneous elicitors are released only by treatments that are injurious to plant cells. The physiological role of endogenous elicitors in plant-pathogen interactions is therefore unclear.

Whatever the causal mechanism, photoalexin elicitation has been shown in several plants to entail the *de novo* transcription and translation of a relatively small number of plant genes (Bell et al., 1984; Chappell and Hahlbrock, 1984; Hadwiger and Wagoner, 1983b; Hahlbrock et al., 1981; Kreuzaler et al., 1983; Kuhn et al., 1984; Schmelzer et al., 1984), and it has been argued that these changes are causally connected with ultimate disease resistance. Some of the inducibly formed mRNAs have been identified as coding for certain biosynthetic enzymes in the phytoalexin biosynthetic pathway of legumes, including phenylalanine ammonia-lyase and chalcone synthase (Bell et al., 1984; Kuhn et al., 1984). As noted in the next section, it will be of interest to perform similar studies when c-DNA probes become available for later enzymes in phytoalexin biosynthetic pathways.

Recent papers have noted similarities between agents which elicit phytoalexin production and other stresses such as a proteinase inhibitor-inducing factor (PIIF) (Walker-Simmons et al., 1983; 1984b). Bishop et al. (1984) identified PIIF from tomato leaves as pectic oligomers which were liberated from injured leaves and systemically moved upward in the plant. Walker-Simmons et al. (1984b) subsequently showed that pectic fragments liberated from higher plant cell walls by a fungal endopolygalacturonase were efficient both as a phytoalexin elicitor and as PIIF. Walker-Simmons et al. (1984a) also found that the activity of PIIF in tomato leaves involved a change in the integrity of the plant plasma membrane. This is similar to the behavior of certain phytoalexin elicitors such as victorin (Scheffer and Livingston, 1984).

Hadwiger and Wagoner (1983a) found that heat-shock stimulated a different class of proteins than did phytoalexin elicitors. Heat shock also blocked the subsequent formation of the defense-related

proteins when an elicitor was supplied. These data argue that different transcriptional events are initiated in plant tissue by heat shock and phytoalexin elicitors.

BIOSYNTHESIS OF PHYTOALEXINS

There has been progress in understanding the molecular mechanisms that ultimately result in phytoalexin accumulation in plant tissues. As discussed above, several groups have shown that phytoalexin accumulation involves the specific production of a relatively small number of specific messenger RNAs rapidly after pathogen infection or elicitor treatment. Much of this work has been done with artificial systems such as suspension cells, and in these the production of phytoalexins seems regulated strictly by synthetic rate. There is considerable evidence that the accumulation of phytoalexins in natural plant tissues inoculated with incompatible pathogens involves an additional factor, inhibited degradation of phytoalexins in dead, hypersensitive plant cells (Sakai et al., 1982; Yoshikawa et al., 1979). Necrotic plant cells therefore appear to provide a reservoir in which phytoalexins accumulate to high levels with minimal degradation by catabolic enzymes located in living cells.

Thus far, few phytoalexins have been synthesized in totally cell free *in vitro* systems (e.g., Sitton and West, 1975). However, information has been steadily accumulating on the biosynthetic pathways leading to several phytoalexins, especially in plants of the Leguminosae. Prof. H. Grisebach's group in particular has made exciting progress with several enzymes in the terminal portion of the glyceollin biosynthetic pathway of soybean. These findings have generally substantiated the pathway as previously deduced by feeding experiments (Banks and Dewick, 1983). Grisebach's group detected two different prenyl transferase enzymes. One of them catalyzes the addition of a prenyl group to glycinol, the immediate precursor of the soybean glyceollins (Zähringer et al., 1979, 1981). The same group also discovered an enzyme that hydroxylates 3,9-dihydroxypterocarpan to form glycinol (Hagmann et al., 1984) and, more recently, made a major discovery when they detected enzymatic activity which converted 4',7-dihydroxyflavanone to the corresponding isoflavone, daidzein (Hagmann and Grisebach, 1984). The aryl migration involved in this reaction has been of interest to plant biochemists for several years, and Grisebach's group now appears to have detected the responsible enzyme. All of the four enzymes above occurred at low or undetectable levels in normal soybean tissue or suspension cells but

were induced by treatment with an appropriate elicitor or infection with an incompatible fungus. The results are therefore consistent with observations that treatments resulting in phytoalexin production elicit the appearance of new messenger RNAs for certain biosynthetic enzymes. The gene cloning and mRNA experiments have thus far been restricted to relatively early enzymes in phytoalexin biosynthetic pathways that also contribute to other secondary metabolites. Grisebach's recent results encourage the thought that genes coding for later enzymes committed solely to biosynthesis of the glyceollins will be cloned in the near future.

APPLICATION OF PHYTOALEXIN RESEARCH

Recombinant DNA technology is revolutionizing our ability to transfer genetically controlled phenotypes from one organism to another, and dramatic recent successes with Ti plasmid derivatives have extended this technology to plants (Broglie et al., 1984; Comai et al., 1983; Horsch et al., 1984). While major problems remain, such as their proper regulation, cloned genes can routinely be incorporated into the chromosomes of a desired plant and are inherited in Mendelian fashion by progeny. The recent development of a "leaf disc technique" by Horsch et al. (1985) for plant transformation promises to further increase the practicality of Ti vectors for introduction of foreign genes into plants. The probability that such Ti vector technology will become routine permits consideration of many possible avenues to improving plant productivity. One of the major of these is the prospect of improving pest and disease control by manipulating genes concerned with disease resistance and phytoalexin biosynthesis.

The considerable amount of basic information gained from phytoalexin studies in the last 15 years places us in the position to attempt using it for practical disease control in the field. Several approaches are possible, including the genetic tailoring of higher plants to make phytoalexin structures that they do not presently produce, alteration of plant response genes such that phytoalexin production is increased following infection with a normally compatible pathogen, and the transfer of disease resistance genes between plants that cannot be intercrossed.

As already discussed, many pathogens which attack single plant species have adapted in various ways to either not elicit, chemically degrade, or tolerate the phytoalexins of the host. The corollary to this observation is that the same pathogens frequently cannot similarly

manage the phytoalexins of other plant species; indeed this may be one of the reasons that they are non-pathogenic on those plants. As additional biosynthetic genes in phytoalexin pathways are cloned, it should be possible to introduce them into foreign plants, thus broadening their arsenal of phytoalexins. For instance, introduction of genes into pea plants for production of the greenbean phytoalexins phaseollin or kievitone might reduce the pathogenicity of *Fusarium solani* f. sp. *pisi* on pea, because, as the groups of VanEtten and Smith have shown, f. sp. *pisi* can degrade and tolerate pisatin but not phaseollin and kievitone. Introduction into pea plants of genes coding for enzymes involved in phaseollin and kievitone production would therefore be expected to confer resistance against *F. solani* f. sp. *pisi*. It should be noted, however, that proper functioning of the introduced genes would likely not be simple. A major problem would be to put them under control of the phytoalexin regulatory machinery of the recipient plant. Since phytoalexins are relatively toxic molecules, as noted earlier, it would be imperative that recipient plants not produce them constitutively. While the desired regulation of introduced genes is currently a major challenge, rapid advances are occurring in our understanding of differential gene expression in eukaryotes (e.g., Broglie et al., 1984; Reudelhuber, 1984).

Plant susceptibility to certain pathogens is frequently due to the inability of the plant to recognize the invader as alien and invoke the HR/phytoalexin response. Naturally, any strategy which broadened the recognitional battery of such plants could be of utility in disease control. As already indicated, one of the mechanisms conferring general disease resistance may be the ability of plants to detect widely conserved structural features of fungal pathogens and insects such as chitin and glucan polymers. If these recognition mechanisms operate through plant receptors, as we believe, then the transfer of a gene or genes coding for the appropriate receptor to defiicient plants might effect disease control. As before, major problems would be posed by the requirements of expression of the receptor gene and location of the receptor protein in the proper cellular location, presumably the plasma membrane. Finally, the correct coupling of receptor function to the specific gene expression associated with phytoalexin induction represents a current uncertainty.

Yet another possible approach to harnessing the phytoalexin mechanism for agricultural benefit involves attempting to increase the magnitude of the phytoalexin response after pathogen challenge. If the localized accumulation of phytoalexins at a pathogen infection site could be made to occur more rapidly and attain greater

magnitude, resistance would likely be attained against currently successful pathogens and pests. Developing such capability would again be an imposing problem both genetically and biochemically. One could presumably increase the enzymatic capacity for phytoalexin synthesis by introducing reiterated copies of existing genes for phytoalexin biosynthesis into plants. However, this might not significantly increase phytoalexin production if the flux of biosynthetic intermediates was not sufficient. It is therefore appealing to think in the long term about increasing the biosynthetic capacity of plant cells for the precise intermediates of pathways such as the shikimate, acetate and mevalonate pathways that are involved in phytoalexin synthesis.

A final approach to harnessing information about phytoalexins for agricultural benefit involves the molecular cloning of disease resistance genes from plants and their introduction into other plants that cannot be conventionally intercrossed with the donors. This might involve not only defined single genes for specific disease resistance but also those little-studied genes that are responsible for general resistance. As I noted earlier, general resistance operates to exclude entire pathogen species from attacking a plant species. We do not currently know the plant genetics of recognition underlying this process, but it would be of interest to transfer such recognitional genes to new plant species, genera, and families in order to see if they would effect general disease resistance in the recipients. However, would the introduced pathogen recognition system interface properly with derepression of phytoalexin biosynthetic genes in the recipient plant? The possibility of failure is tempered by the considerable potential gain if such an experiment were successful. If genes conferring general resistance could be transferred to new plants, they would be expected to function as a relatively permanent disease control measure. A major problem with the current use of specific resistance genes is the evolution of virulent pathogen races, as noted previously. General resistance, however, is not affected by the evolution of virulent pathogen races, at least in the evolutionary short term. Accordingly, if general resistance was transferred to new plants, we would expect many years of stable disease or pest control.

ACKNOWLEDGMENT

The author's research is supported by grants from the National Science Foundation.

LITERATURE CITED

Allen, E. H. and C. A. Thomas. 1971. *Trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol, an antifungal polyacetylene from diseased safflower (*Carthamus tinctorius*). Phytochemistry 10:1579-1582.

Anderson, A. J. 1980. Differences in the biochemical compositions and elicitor activity of extracellular components produced by three races of a fungal plant pathogen, *Colletotrichum lindemuthianum*. Can. J. Microbiol. 26:1473-1479.

Bailey, J. A. 1982. Physiological and biochemical events associated with the expression of resistance to disease, pp. 39-65. *In*: R. K. S. Wood (ed.). Active defense mechanisms in plants. Plenum Press, New York.

———— and J. W. Mansfield. 1982. Phytoalexins. J. Wiley, New York.

Bakker, J. and collaborators. 1983. Photoactivation of isoflavonoid phytoalexins: involvement of free radicals. Photochem. Photobiol. 38:323-329.

Banks, S. W. and P. M. Dewick. 1983. Biosynthesis of glyceollins I, II and III in soybean. Phytochemistry 22:2729-2733.

Bell, J. N. and collaborators. 1984. Differential induction of chalcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant-pathogen interactions. Proc. Natl. Acad. Sci. USA 81:3384-3388.

Biggs, D. R., G. A. Lane, G. B. Russell, and O. R. W. Sutherland. 1983. Insect feeding deterrent activity of isoflavonoid phytoalexins. Fourth Int. Congr. Plant Pathol. Abstract 700. Melbourne, Australia.

Bishop, P. D., G. Pearce, J. E. Bryant, and C. A. Ryan. 1984. Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. Identity and activity of poly- and oligogalacturonide fragments. J. Biol. Chem. 259: 13172-13177.

Bouillant, M-L., J. Favre-Bonvin, and P. Ricci. 1983. Dianthalexine, nouvelle phytoalexine de type benzoxazinone, isolee de l'oeillet *Dianthus caryophyllus* L. (*Caryophyllacees*). Tetrahedron Letters 24:51-52.

Boydston, R., J. D. Paxton, and D. E. Koeppe. 1983. Glyceollin: a site-specific inhibitor of electron transport in isolated soybean mitrochondria. Plant Physiol. 72: 151-155.

Broglie, R. and collaborators. 1984. Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plant cells. Science 224:838-843.

Bruce, R. J. and C. A. West. 1982. Elicitation of casbene synthetase activity in castor bean. The role of pectic fragments of the plant cell wall in elicitation by a fungal endopolygalacturonase. Plant Physiol. 69:1181-1188.

Bufler, G. and F. Bangerth. 1982. UV-induced peroxidase and phenylalanine ammonia-lyase activity and phaseollin accumulation in leaves of *Phaseolus vulgaris* L. in relation to ethylene. Plant Sci. Letters 25:227-237.

Chappell, J. and K. Hahlbrock. 1984. Transcription of plant defence genes in response to UV light or fungal elicitor. Nature 311:76-78.

————, ————, and T. Boller. 1984. Rapid induction of ethylene biosynthesis in cultured parsley cells by fungal elicitor and its relationship to the induction of phenylalanine ammonia-lyase. Planta 161:475-480.

Comai, L., C. Schilling-Cordaro, A. Mergia, and C. M. Houck. 1983. A new technique for genetic engineering of *Agrobacterium* Ti plasmid. Plasmid 10:21-30.

Darvill, A. G. and P. Albersheim. 1984. Phytoalexins and their elicitors—a defense against microbial infection in plants. Annu. Rev. Plant Physiol. 35:243-275.

Davis, K. R., G. D. Lyon, A. G. Darvill, and P. Albersheim. 1984. Host-pathogen interactions. XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant Physiol. 74:52-60.

De Wit, P. J. G. M., A. E. Hofman, G. C. M. Velthuis, and J. A. Kuć. 1985. Isolation and characterization of an elicitor of necrosis isolated from intercellular fluids of compatible interactions of *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato. Plant Physiol. 77:642-647.

Dixon, R. A., P. M. Dey, M. A. Lawton, and C. J. Lamb. 1983. Phtoalexin induction in French bean. Intercellular transmission of elicitation in cell suspension cultures and hypocotyl sections of *Phaseolus vulgaris*. Plant Physiol. 71:251-256.

Gabriel, D. W. 1984. Molecular cloning of specific avirulence genes from *Xanthomonas malvacearum*. Proc. 2nd Inter. Symp. on the Molecular Genetics of the Bacterial-Plant Interaction (in press).

Grisebach, H. 1983. Phytoalexins. Mycol. Ser. 5:377-428.

————, H. Börner, and P. Moesta. 1982. Zur induktion der Phytoalexin synthese bei *Soja* und ihre Bedeutung für die Resistenz genenüber *Phytophthora megasperma* f. sp. *glycinea*. Ber. Deutsch Bot. Ges. 95:619-642.

Hadwiger, L. A. and J. M. Beckman. 1980. Chitosan as a component of pea-*Fusarium solani* interactions. Plant Physiol. 66:205-211.

———— and W. Wagoner. 1983a. Effect of heat shock on the mRNA-directed disease resistance response of peas. Plant Physiol. 72:553-556.

———— and ————. 1983b. Electrophoretic patterns of pea and *Fusarium solani* proteins synthesized *in vitro* or *in vivo* which characterize the compatible and incompatible interactions. Physiol. Plant Pathol. 23:153-162.

Hagmann, M. and H. Grisebach. 1984. Enzymatic rearrangement of flavanone to isoflavone. Federated European Biological Societies Letters 175:199-202.

Hagmann, H-L., W. Heller, and H. Grisebach. 1984. Induction of phytoalexin synthesis in soybean. Stereospecific 3,9-dihydroxypterocarpan 6a-hydroxylase from elicitor-induced soybean cell cultures. European J. Biochemistry 142:127-131.

Hahlbrock, K. and collaborators. 1981. Rapid response of suspension cultured parsley cells to the elicitor from *Phytophthora megasperma* var. *sojae*.

Induction of the enzymes of general phenylpropanoid metabolism. Plant Physiol. 67:768-773.

Hahn, M. G. and H. Grisebach. 1983. Cyclic AMP is not involved as a second messenger in the response of soybean to infection by *Phytophthora megasperma* f. sp. *glycinea*. Z. Naturforsch. 38c:578-582.

————, A. Bonhoff, and H. Grisebach. 1985. Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol. 77: 591-601.

Harder, D. E. and collaborators. 1979. Electron microscopy of susceptible and resistant near-isogenic (*sr6/Sr6*) lines of wheat infected by *Puccinia graminis tritici*. II. Expression of incompatibility in mesophyll and epidermal cells and the effect of temperature on host-parasite interactions in these cells. Can. J. Bot. 57:2617-2625.

Hargreaves, J. A. 1981. Accumulation of phytoalexins in cotyledons of French bean (*Phaseolus vulgaris* L.) following treatment with Triton (T-octylphenol polyethoxyethanol) surfactants. New Phytol. 87:733-741.

Hart, S. V., M. Kogan, and J. D. Paxton. 1983. Effect of soybean phytoalexins on the herbivorous insects Mexican bean beetle and soybean looper. J. Chem. Ecol. 9:657-672.

Holliday, M. J. and W. L. Klarman. 1979. Expression of disease reaction types in soybean callus from resistant and susceptible plants. Phytopathology 69:576-578.

Horsch, R. B. and collaborators. 1984. Inheritance of functional foreign genes in plants. Science 223:496-498.

———— and collaborators. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.

Ingham, J. L. 1982. Phytoalexins from the Leguminosae, pp. 21-80. *In*: J. A. Bailey and J. W. Mansfield (eds.). Phytoalexins. John Wiley, New York.

Kaplan, D. T., N. T. Keen, and I. J. Thomason. 1980. Studies on the mode of action of glyceollin soybean incompatibility to the root knot nematode, *Meloidogyne incognita*. Physiol. Plant Pathol. 16:319-325.

Keen, N. T. 1981. Evaluation of the role of phytoalexins, pp. 155-177. *In*: R. Staples and G. Toenessien (eds.). Plant Disease Control. J. Wiley, New York.

————. 1982. Specific recognition in gene-for-gene host-parasite systems. Adv. Plant Pathol. 1:35-82.

————. 1985. Progress in understanding the biochemistry of race-specific interactions, pp. 85-101. *In*: J. Grothond W. Bushnell (eds.). Genetic basis of biochemical mechanisms of plant disease. APS Press, St. Paul.

———— and B. Bruegger. 1977. Phytoalexins and chemicals that elicit their production in plants. Amer. Chem. Soc. Symp. Ser. 62:1-26.

————, M. J. Holliday, and M. Yoshikawa. 1982. Effects of glyphosate on glyceollin production and the expression of resistance to *Phytophthora megasperma* f. sp. *glycinea* in soybean. Phytopathology 72:1467-1470.

———, R. L. Lyne, and T. Hymowitz. 1986. Phytoalexin production as a chemosystematic parameter within the genus *Glycine* (Leguminosae, Papilionoideae). Biochem. Syst. Ecol. (in press).

Kistler, H. C. and H. D. VanEtten. 1984. Regulation of pisatin demethylation in *Nectria haematococca* and its influence on pisatin tolerance and virulence. J. Gen. Microbiol. 130:2605-2613.

Kreuzaler, F. and collaborators. 1983. UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc. Natl. Acad. Sci. USA 80:2591-2593.

Kuć, J. 1982. Phytoalexins from the Solanaceae, pp. 81-105. *In*: J. A. Bailey and J. W. Mansfield (eds.). Phytoalexins. J. Wiley, New York.

Kuhn, D. N., J. Chappell, A. Boudet, and K. Hahlbrock. 1984. Induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor. Proc. Nat. Acad. Sci. USA 81:1102-1106.

Langcake, P. and R. J. Pryce. 1977. A new class of phytoalexins from grapevines. Experientia 33:151-152.

Lazarovits, G., P. Stössel, and E. W. B. Ward. 1981. Age-related changes in specificity and glyceollin production in the hypocotyl reaction of soybeans to *Phytophthora megasperma* var. *sojae*. Phytopathology 71:94-97.

Long, M. and collaborators. 1985. Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Phytopathology 75:235-239.

Lyon, G. D. 1984. A note on the effect of temperature on the sensitivity of *Erwinia carotovora* sub sp. *atroseptica* to the phytoalexin rishitin. J. Appl. Bacteriol. 57:191-192.

Mansfield, J. W. 1983. Antimicrobial compounds, pp. 237-265. *In*: J. A. Callow (ed.). Biochemical plant pathology. J. Wiley, New York.

Mayama, S. and N. T. Keen. 1984. Production of victorin by various isolates of *Helminthosporium victoriae* and its phytoalexin elicitor activity. Phytopathology 74:850 (Abstr.).

——— and collaborators. 1982a. Effects of elevated temperature and α-aminooxyacetate on the accumulation of avenalumins in oat leaves infected with *Puccinia coronata* f. sp. *avenae*. Physiol. Plant Pathol. 20:305-312.

———, Y. Matsuura, H. Iida, and T. Tani. 1982b. The role of avenalumin in the resistance of oat to crown rust, *Puccinia coronata* f. sp. *avenae*. Physiol. Plant Pathol. 20:189-199.

——— and collaborators. 1981. Isolation and structure elucidation of genuine oat phytoalexin, avenalumin I. Tetrahedron Letters 22:2103-2106.

Moesta, P. and H. Grisebach. 1981. Investigation of the mechanism of phytoalexin accumulation in soybean induced by glucan or mercuric chloride. Arch. Biochem. Biophys. 211:39-43.

———— and ————. 1982. L-2-aminooxy-3-phenylpropionic acid inhibits phytoalexin accumulation in soybean with concomitant loss of resistance against *Phytophthora megasperma* f. sp. *glycinea*. Physiol. Plant Pathol. 21:65-70.

————, M. G. Hahn, and H. Grisebach. 1983. Development of a radioimmunoassay for the soybean phytoalexin glyceollin I. Plant Physiol. 73:233-237.

————, U. Seydel, B. Lindner, and H. Grisebach. 1982. Detection of glyceollin on the cellular level in infected soybean by laser microprobe mass analysis. Z. Naturforsch. 37c:748-751.

Nakajima, T., K. Tomiyama, and M. Kinukawa. 1975. Distribution of rishitin and lubimin in potato-tuber tissue infected by an incompatible race of *Phytophthora infestans* and the site where rishitin is synthesized. Ann. Phytopathol. Soc. Japan 41:49-55.

Paradies, I., J. R. Konze, E. F. Elstner, and J. Paxton. 1980. Ethylene: indicator but not inducer of phytoalexin synthesis in soybean. Plant Physiol. 66:1106-1109.

Pankhurst, C. E. and D. R. Biggs. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. 26:542-545.

Pueppke, S. G. 1978. Some intriguing aspects of the putative role of isoflavonoid phytoalexins in plant disease. Mycopathologia 65:115-119.

Reudelhuber, T. 1984. Upstream and downstream control of eukaryotic genes. Nature 312:700-701.

Russell, G. B., O. R. W. Sutherland, R. F. N. Hutchins, and P. E. Christmas. 1978. Vestitol: a phytoalexin with insect feeding-deterrent activity. J. Chem. Ecol. 4:571-579.

Sakai, S., N. Doke, and K. Tomiyama. 1982. Relation between necrosis and rishitin accumulation in potato tuber slices treated with hyphal wall components of *Phytophthora infestans*. Ann. Phytophathol. Soc. Japan 48:238-240.

Scheffer, R. P. and R. S. Livingston. 1984. Host-selective toxins and their role in plant diseases. Science 223:17-21.

Schmelzer, E. and collaborators. 1984. Phytoalexin synthesis in soybean (*Glycine max*). Similar time courses of mRNA induction in hypocotyls infected with a fungal pathogen and in cell cultures treated with fungal elicitor. Federated European Biological Societies Letters 172:59-63.

Sharp, J. K., M. McNeil, and P. Albersheim. 1984. The primary structures of one elicitor-active and seven elicitor-inactive hexa(β-D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f. sp. *glycinea*. J. Biol. Chem. 259:11321-11336.

Sitton, D. and C. A. West. 1975. Casbene: an anti-fungal diterpene produced in cell-free extracts of *Ricinus communis* seedlings. Phytochemistry 14:1921-1925.

Smith, D. A., H. E. Wheeler, S. W. Banks, and T. E. Cleveland. 1984. Association between lowered kievitone hydratase activity and reduced virulence to bean in variants of *Fusarium solani* f. sp. *phaseoli*. Physiol. Plant Pathol. 25:135-147.

Smith, I. M. 1980. Phytoalexines: a revue. Ann., Phytopathol. 12:321-328.

Staskawicz, B. J., D. Dahlbeck, and N. T. Keen. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Nat. Acad. Sci. USA 81:6024-6028.

Sutherland, O. R. W., G. B. Russell, D. R. Biggs, and G. A. Lane. 1980. Insect feeding deterrent activity of phytoalexin isoflavonoids. Biochem. Syst. Ecol. 8:73-75.

Sweigard, J. A. and H. D. VanEtten. 1984. Polar lipids from pea reduce the pisatin sensitivity of *Aphanomyces euteiches*. Phytopathology 74:833 (Abstr.).

Tegtmeier, K. J. and H. VanEtten. 1982. The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: a genetic analysis. Phytopathology 72:608-612.

Tietjen, K. and U. Matern. 1984. Induction and suppression of phytoalexin biosynthesis in cultured cells of sunflower, *Carthamus tinctorius* by metabolites of *Alternaria carthami*. Arch. Biochem. Biophys. 229:136-144.

VanEtten, H. D. and J. I. Stein. 1978. Differential response of *Fusarium solani* isolates to pisatin and phaseollin. Phytopathology 68:1276-1283.

———— and S. G. Pueppke. 1976. Isoflavonoid phytoalexins, pp 239-289. *In*: J. Friend and D. Threlfall (eds.). Biochemical aspects of plant-parasite relationships. Academic Press, London.

Walker-Simmons, M., L. Hadwiger, and C. A. Ryan. 1983. Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem. Biophys. Res. Comm. 110:194-199.

————, H. Holländer-Czytko, J. K. Anderson, and C. A. Ryan. 1984a. Wound signals in plants: a systemic plant wound signal alters plasma membrane integrity. Proc. Natl. Acad. Sci. USA 81:3737-3741.

———— and collaborators. 1984b. Comparison of proteinase inhibitor-inducing activities and phytoalexin elicitor activities of a pure fungal endopolygalacturonase, pectic fragments, and chitosans. Plant Physiol. 76:833-836.

Ward, E. W. B. and G. Lazarovits. 1982. Temperature-induced changes in specificity in the interaction of soybeans with *Phytophthora megaspermas* f. sp. *glycinea*. Phytopathology 72:826-830.

Weinstein, L. I. and P. Albersheim. 1983. Host-pathogen interactions. XXIII. The mechanism of the antibacterial action of glycinol, a pterocarpan phytoalexin synthesized by soybeans. Plant Physiol. 72:557-563.

Yoshikawa, M. 1983. Macromolecules, recognition, and the triggering of resistance, pp. 267-298. *In*: J. A. Callow (ed.). Biochemical plant pathology. J. Wiley, New York.

————, N. T. Keen, and M-C Wang. 1983. A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. Plant Physiol. 73:497-506.

————, K. Yamauchi, and H. Masago. 1978a. *De novo* messenger RNA and protein synthesis are required for phytoalexin-mediated disease resistance in soybean hypocotyls. Plant Physiol. 61:314-317.

———, ———, and ———. 1978b. Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infectd with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 12:73-82.

———, ———, and ———. 1979. Biosynthesis and biodegradation of glyceollin by soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 14:157-169.

Zähringer, U. and collaborators. 1979. Induction of phytoalexin synthesis in soybean. Dimethylallylpyrophosphate: trihydroxypterocarpan dimethylallyl transferase from elicitor-induced cotyledons. Federated European Biological Societies Letters 101:90-92.

———, E. Schaller, and H. Grisebach. 1981. Induction of phytoalexin synthesis in soybean. Structure and reactions of naturally occurring and enzymatically prepared prenylated pterocarpans from elicitor-treated cotyledons and cell cultures of soybean. Z. Naturforsch. 36c:234-241.

IOWA STATE JOURNAL OF RESEARCH / May, 1986 501-527
Vol. 60, No. 4

NATURAL CHEMICALS IN PLANT RESISTANCE TO INSECTS

Marcos Kogan[1]

ABSTRACT. Breeding for insect resistance has often advanced without the benefit of knowledge of underlying biochemical mechanisms. Examples derived from insects associated with representative plants in the Solanaceae, Cruciferae and Leguminosae illustrate the allelochemical role of secondary plant metabolites in host-plant selection and in resistance processes. Using those three herbivore/plant systems as models, it is demonstrated that benefit for host plant resistance breeding from basic information on insect/plant interactions will be realized from an interpretation of the biochemical processes in a broad ecological context. The goal of maximizing yield often clashes with the maintenance of adequate defensive postures in many crop cultivars. A better understanding of mechanisms may help set realistic goals of breeding cultivars with high yields that maintain a reasonable defense capability.

Index descriptors: Insect/plant interactions, secondary plant compounds, coevolution, herbivory, soybean, Cruciferae, Solanaceae, *Glycine max*.

INTRODUCTION

Most living plants, cultivated or wild, have some degree of resistance against most insects and probably also against most pathogens. Otherwise, we would have to accept a more barren landscape than the one that surrounds us. The susceptibility of agricultural plants to pests is a question of a plant's degree of resistance and spectrum of the resistance profile versus the range of potential challengers. Susceptibility is further defined against the level of injury that we accept as tolerable within the economic constraints of the crop. Injury to a few blossoms of soybean may be irrelevant to final yield, but a blemish on a gladiola flower is a total loss.

In general, plant defense mechanisms (= resistance) against herbivores are multidimensional. Chemical defenses are only part of a complex strategy that permits plants to coexist with herbivores, ward off pathogen attacks, and successfully compete with other plant species in the same community. Only within the global ecological

[1]Illinois Natural History Survey and University of Illinois at Urbana-Champaign. Section of Economic Entomology, 172 Natural Resources Building, 607 E. Peabody Dr., Champaign, IL 61820.

context of the community processes may we fully appreciate the role of chemical defenses and learn how to use them in crop protection.

Relatively few crop plants account for much of the food and fiber required for survival of humans on this planet. Seven major crops (wheat, *Triticum* spp.; rice, *Oryza sativa* L.; potato, *Solanum tuberosum* L.; maize, *Zea mays* L.; dry beans, *Phaseolus vulgaris* L.; soybean, *Glycine max* (L.) Merrill; and cotton, *Gossypium* spp.) are grown on 44% of the total arable land and land under permanent crops in the world (FAO, 1981). These plants originated in a few centers of diversity (Harlan, 1975) from which they spread into all areas of modern cultivation. On the average, 67% of the area cultivated to those seven crops falls outside the continent where they presumably originated. When introduced into new areas, crop plants were exposed to diverse ecological conditions, new faunas, weed competitors and pathogens all of which often stretched plants to the limit of their adaptability and increased pressures on their defensive capabilities. Pest problems often intensify for plants that are grown in the fringes of their ecological tolerances, because of the expenditure of energy needed to overcome such stress factors as heat, drought, and nutrient imbalances that lower the plant's resistance to pests. For example, the soybean, a temperate zone crop, is relatively free of major insect pests both in Northeastern China, where it originated, and in midwestern United States, where it has been intensively grown for less than 75 years (Kogan, 1980; 1981). Nevertheless, insect pests are a greater problem for soybean in the southern latitudes of these countries. In Northern Florida, for example, environmental conditions are favorable for potential pests and often marginal for soybean, so that outbreaks of the velvetbean caterpillar (*Anticarsia gemmatalis*) occur every year and require several insecticide applications (Kogan, 1980).

The search for pest resistant crop plants and breeding for resistant cultivars has advanced for years without great concern for the underlying biochemical causes (Painter, 1951; Chapman and Bernays, 1977; Hedin et al., 1977; Maxwell, 1977; Harris, 1980; Norris and Kogan, 1980). In the last two decades, increasing research in the basic processes of insect/plant interactions is helping to explain the mechanisms of resistance in crop plants. The picture that emerges is much more complex than previously conceived.

Books on various aspects of insect/plant relationships have emerged at a rate of about two per year over the past ten years, while several hundred research papers are added yearly to the literature (Kogan, in press). Much of this literature deals with chemical aspects

of insect/plant interactions. Thus, I will not attempt a comprehensive review of the literature of the field. Instead, in this paper I briefly discuss the nature and roles of secondary plant compounds and the concept of coevolution, and I will then use three plant/insect systems as paradigms of the kinds of relationships mediated by these compounds. These systems illustrate a range of natural-product chemistry and various aspects of insect adaptations to those natural products. The three systems include representative examples in the following families: Solanaceae (potato and tomato), Cruciferae (various), and Leguminosae (mainly common bean and soybean). In conclusion I deal with the possible use in plant-resistance breeding of information on the anti-herbivory role of secondary metabolites.

SECONDARY PLANT COMPOUNDS

Some of the most ecologically active plant compounds are secondary metabolites. However, the distinction between primary and secondary plant compounds is far from absolute, either chemically (Mothes, 1980) or ecologically. Whereas primary metabolites (carbohydrates, amino acids, purine and pyrimidine bases, and lipids) are ubiquitous and perform vital roles in all living organisms, the secondary metabolites (alkaloids, terpenes, flavonoids, steroids, tannins, etc.) have more limited distribution among organisms and frequently have little obvious survival value outside an ecological context.

Although the physiological role of many secondary plant compounds is still obscure, their ecological function is currently recognized even by nonecologically oriented organic chemists (see chapters in Bell and Charlwood, 1980). The diversity, multiplicity, and unique patterns of occurrence of secondary plant compounds and the patterns of host preferences by oligophagous insects have formed the bases of current theories of insect/plant interactions and coevolution (Dethier, 1954, 1970; Fraenkel, 1959, 1969; Thorsteinson, 1960; Ehrlich and Raven, 1964; Kogan, 1977; Hedin, 1977, 1983; Rosenthal and Janzen, 1979; Harborne, 1982; Crawley, 1983; Futuyma and Slatkin, 1983; Kogan, in press).

Taxonomically related plants often have similar natural product chemistry. Some of these patterns have been used successfully in chemotaxonomic studies (Bisby et al., 1980). However, it is not uncommon for unrelated plant groups to have similar secondary metabolites (see the Cruciferae system below).

In summary, the unique secondary chemical makeup of plants explains many close host associations of plant feeding insects. Oligophagous species tend to respond specifically to compounds that induce feeding or oviposition in the insect. These same or other compounds act as repellents, feeding or oviposition deterrents, or antibiotics against most other sympatric phytophagous species. The evolutionary processes that selected for specific defensive natural-product biosynthesis in plants and the counteradaptive reversal of defensive chemicals into attractants and feeding excitants in some phytophagous insects have been defined as coevolution (Ehrlich and Raven, 1964).

COEVOLUTION

The patterns of host specificity observed in contemporary insect faunas are the result of adaptive evolutionary processes. However, not all interactive systems are necessarily coevolutionary (Janzen, 1980). Some argue that few, if any, insect/plant antagonistic systems result from direct coevolutionary interactions. Most insect/plant associations probably resulted from secondary adaptations of insects to resources with defensive strategies evolved under selection pressures from quite different antagonists. As more is known about the similarities of chemical mechanisms of resistance against insects and pathogens, it becomes apparent that herbivores and pathogens are concurrent factors in the plant's chemical evolution. For example, DIMBOA, known for almost 20 years as an antibiotic factor in maize against the European corn borer, was recently found to be an important resistance factor against the fungus *Helminthosporium turcicum* in several inbred lines (Toldine, 1984).

To be coevolution, an evolutionary change in a trait of species A (a host plant) must be followed by a change in a species B (an herbivore) that allows B to overcome the inadequacy temporarily caused by the new plant trait. The process may repeat itself in successive cycles (see Strong et al., 1984 for a discussion of this model).

Within the three systems discussed in this paper, insects and plants have not necessarily coevolved. The arthropods on crucifers was used by Strong et al. (1984) as an example of a system that could be explained by evolutionary processes other than coevolution. The coevolutionary model should, therefore, be kept within the parameters apparently intended by Ehrlich and Raven (1964) and more formally specified by later critics (Janzen, 1980; Futuyma, 1983; Strong et al., 1984).

ALLELOCHEMICAL ACTIVITY OF SECONDARY PLANT COMPOUNDS

Secondary plant compounds that are produced by one organism and affect other organisms of the same or of a different species are known as allelochemicals. Allelochemicals are allomones if their activity favors the producing organism, and they are kairomones if their activity favors the receiving organism. There are various classifications of the allelochemical effect of secondary plant compounds on insect behavior, development, growth, survival, fecundity and fertility. The system that I adopted (Table 1) combines the classification proposed by Whittaker and Feeny (1971) with the categories of plant resistance to insects commonly used by workers in this field (Beck, 1965; Kogan and Ortman, 1978; Horber, 1980).

The anti-herbivory theory of coevolution suggests that most secondary plant compounds have evolved to perform allomonal functions in a defensive mode. The reciprocal adaptation of specialist herbivores has resulted not only in the evolution of mechanisms that render the compound ineffectual (e.g., detoxification and behavioral avoidance) but also in converting allomones into kairomones, or cues for host-finding, and feeding or oviposition excitation. Consequently, many secondary plant compounds are defensive allomones for most sympatric herbivorous fauna, and they are kairomones for a few oligophages on the allelochemic-producing plant (Norris and Kogan, 1980).

As the taxonomic position of plants and their phytochemical characteristics are closely related, the classification of insects' feeding habits has been generally based on a "dietary taxonomy." Thus monophagous, oligophagous, and polyphagous insects were considered insects associated with one or a few species of plants in a genus, a few genera in a family, or several different families, respectively. Because host associations seem to be governed by the presence or absence of secondary plant compounds, these feeding habits are better functionally defined on the allomone/kairomone characteristic balance within the potential host range of the herbivore. Figure 1 schematizes these relationships.

From these relationships I will attempt to demonstrate that (a) the chemically mediated mechanisms of insect/plant interactions are diverse and involve different defensive strategies by plants and counter-adaptive strategies by insects; and (b) knowledge of co-adaptive mechanisms under natural conditions (non-agricultural systems) helps us understand resistance mechanisms in crop plants. The discussion is limited to chemical defenses against foliage-feeding insects.

Table 1. Principal classes of chemical plant factors (allelochemics) and the corresponding behavioral or physiological effects on insects.[a]

ALLELOCHEMICAL FACTORS	BEHAVIORAL OR PHYSIOLOGICAL EFFECTS
ALLOMONES	Give adaptive advantage to the producing organism
Antixenotics	Disrupt normal host selection behavior
Repellents	Orient insects away from plant
Locomotory excitants	Start or speed up movement
Suppressants	Inhibit biting or piercing
Deterrents	Prevent maintenance of feeding or oviposition
Antibiotics	Disrupt normal growth and development of larvae; reduce longevity and fecundity of adults
KAIROMONES	Give adaptive advantage to the receiving organism
Attractants	Orient insects toward host plant
Arrestants	Slow down or stop movement
Feeding or oviposition excitants	Elicit biting, piercing, or oviposition; promote continuation of feeding

[a](From Kogan, 1982 adapted from Dethier et al., 1960; Beck, 1965; and Whittaker and Feeney, 1971).

DEFENSIVE PATTERNS IN THE SOLANACEAE

A first line of defensive chemistry in the Solanaceae are compounds contained externally in glandular trichomes. Foliar trichomes of the wild Bolivian potato species *Solanum berthaultii* Hawkes are of two types: (a) short trichomes topped by a four-lobed gland, and (b) long trichomes topped by a single ovoid gland (Gibson,

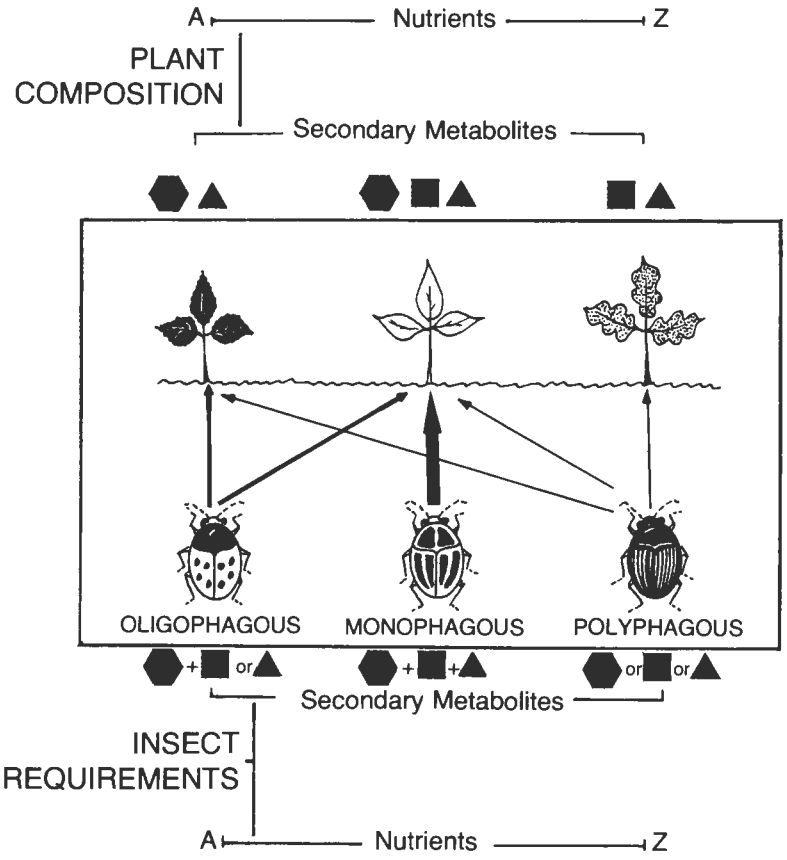
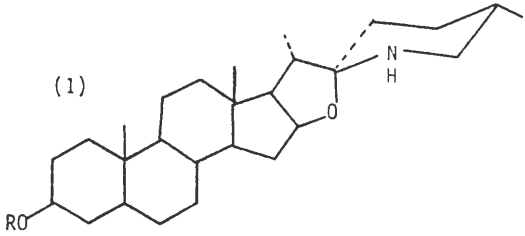


Figure 1. Classes of feeding specialization in insects based on phytochemical characteristics of host plants. Requirements for nutrients are assumed uniform (insect requirements A-Z for the phytophagous insects in all three classes). Plants are assumed similar in nutrient content (plant nutrients A-Z). Plants have different phytochemical profiles represented by combinations of geometric figures (hexagon, square, and triangle). The monophagous insect only accepts plants having the complete profile (hexagon + square + triangle); thus, only one plant satisfies this requirement in the diagram. The oligophagous insect requires the hexagon plus either one of the remaining "phytochemicals" (two plants satisfy this requirement). The polyphagous insect accepts plants that have at least one of the three "phytochemicals" (all three plants satisfy this requirement). The plants may or may not be taxonomically related. The diagram suggests that the phytochemical characteristics govern food specialization, not necessarily taxonomic affinity.

1971). When the glands of type A trichomes are ruptured, the exudate darkens and hardens. Type B trichome exudate remains clear and viscous. Small insects and mites are trapped by these exudates (Tingey and Gibson, 1978). Researchers at Cornell University are attempting to use *S. tuberosum* × *S. berthaultii* hybrids to improve resistance to aphids and leafhoppers (Ryan et al., 1983). Apparently both types of trichomes are required for maximum resistance. The mechanism seems to involve complex reactions mediated by polyphenol oxidases and peroxidase acting on phenolic substrates contained in different compartments in the trichomes (Tingey and Sinden, 1982).

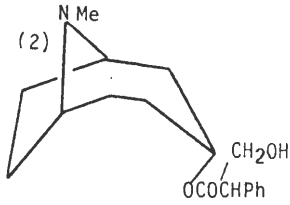
Similarly, glandular trichomes of the wild tomato relative, *Lycopersicon hirsutum* f. *glabratum*, contain large amounts of the keto-hydrocarbon, 2-tridecanone ($CH_3(CH_2)_{10}COCH_3$). This compound seems to have powerful antibiotic action against the tomato fruitworm, *Heliothis zea* (Boddie), and the tobacco hornworm (Williams et al., 1980; Bordner et al., 1983). Also part of the glandular trichome defensive system in tomato is the flavonol glycoside, rutin (5), which, if introduced in artificial media, inhibits growth of *H. zea* larvae (Isman and Duffey, 1980). About one third of the total phenolic content in tomato leaves is stored in the trichomes (Harborne, 1982).

A phytochemical characteristic of both cultivated and wild Solanaceae is the presence of alkaloids: steroidal alkaloids (1) in the genera *Solanum* and *Lycopersicon* (Gregory, 1984); tropane alkaloids (2) in the genera *Datura*, *Hyoscyamus*, and *Atropa*, among others; and pyrrolidine alkaloids (3), such as nicotine in the genus *Nicotiana*. Alkaloids represent a second line of defense in the Solanaceae.



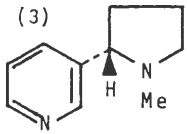
Steroidal alkaloids:

R = H, tomatidine
R = Xyl-2glu-gal, α-tomatine



Tropane alkaloid:

Hyoscyamine



Pyrrolidine alkaloid:

(-)-2'*S*-Nicotine

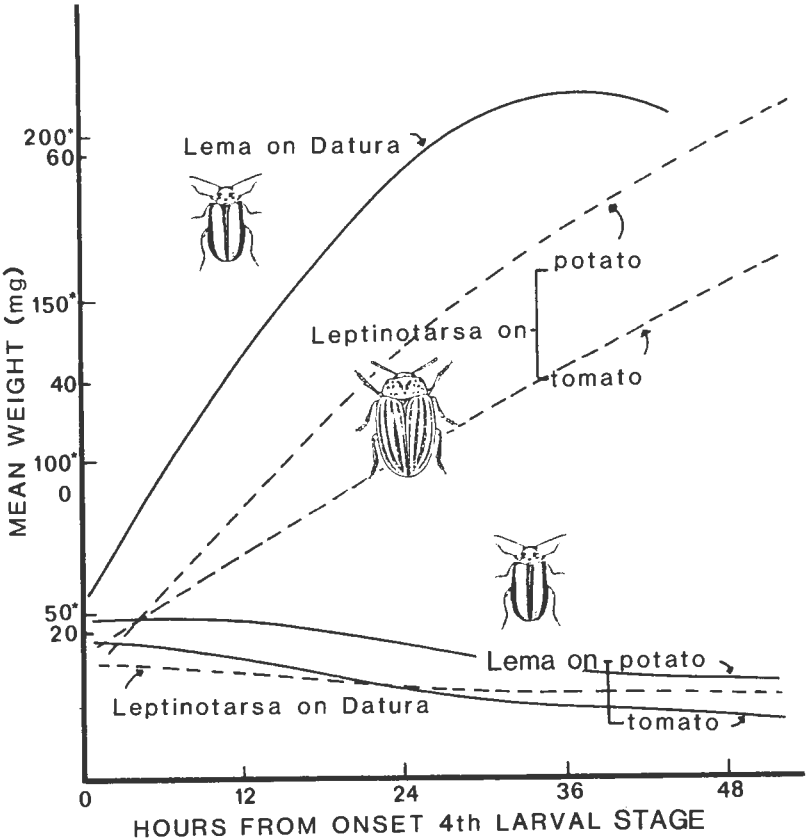
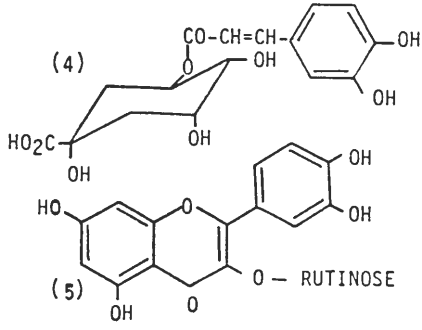


Figure 2. Weight variation of larvae of *Lema trilineata daturaphila* and *Leptinotarsa decemlineata* fed leaves of *Datura meteloides*, potato (*Solanum tuberosum*) or tomato (*Lycopersicon esculentum*), during the fourth instar. The scale numbers followed by an asterisk refer to *Leptinotarsa*. The beetle species seem to have differentially adapted to plants according to prevalent alkaloidal content (see text). (Redrawn from Kogan and Goeden, 1971).

Comparative studies of host preferences of Solanaceae-associated arthropods suggest that the kinds of alkaloids present in a given plant may influence the suitability of the plant for the arthropod. The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), grows well on potato but poorly on tomato or *Datura* foliage (Hsiao and Fraenkel, 1968b). The three-lined potato beetle, *Lema trilineata* (Olivier), on the other hand, grows normally on *Datura* and related genera but performs poorly on potato and tomato (Kogan and Goeden, 1971) (Figure 2). The tobacco hornworm, *Manduca sexta*

(Johannson), by contrast, readily feeds and develops well on potato, tomato, and *Datura*, as well as on tobacco plants (Yamamoto and Fraenkel, 1960).

Feeding excitants for all those phytophagous insects apparently are compounds commonly found in the Solanaceae. These compounds have not been totally characterized, however. Hsiao and Fraenkel (1968a) have suggested that chlorogenic acid (4) is involved in feeding excitation in the Colorado potato beetle, and some excitatory response to this compound was recorded with *Lema trilineata* (Kogan and Goeden, 1971).



Chlorogenic acid

Rutin

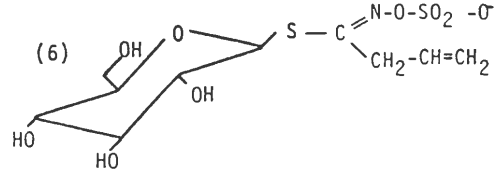
This system may conform with a model proposed by Jermy (1966). Host acceptance would be governed by specific patterns of antibiotic and antixenotic compounds (the steroidal, tropane, or pyrrolidine alkaloids), and feeding excitation would result from nonspecific compounds.

A third level of defense has been suggested by C. A. Ryan and co-workers (Ryan, 1973, 1979; Nelson et al., 1983) as an induced mechanism. Mechanical injury to a single tomato or potato leaf results in the synthesis and accumulation of two proteinase inhibitors in the leaf cells. If within a few hours the leaf is wounded again, inhibitors accumulate at a rate 2-3 times greater than the accumulation rate following the first wounding. Wounding releases a so-called "proteinase inhibitor inducing factor" (PIIF) that travels within the plant and increases response rate to subsequent injury. Current assumption is that PIIF is a pectic polysaccharide associated with the plant cell wall (Nelson et al., 1983). The biochemical aspects of this system have been known for some time, but only recently has the anti-herbivory role of proteinase inhibitor accumulation in tomato been tested experimentally (Broadway et al., in press).

In summary, solanaceous plants may have a vesture of glandular trichomes that contain an array of secondary metabolites: phenolic acids, polyphenols, rutin, and 2-tridecanone. Exudates of this vesture are effective antibiotic and antixenotic factors against lepidopterous larvae, the Colorado potato beetle, flea beetles, aphids, leafhoppers, and spider mites. A post-ingestive line of defense are the glycol-, tropane, and other alkaloids, some of which are potent feeding deterrents. Finally, accumulation of proteinase inhibitors may be induced by mechanical injury to the leaves.

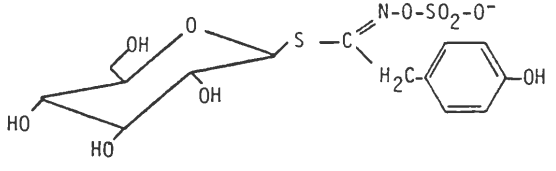
Effective chemical defenses occur in wild relatives of present cultivars. The cultivars seem to have lost many of their defensive capabilities through selective breeding. Whether breeders will be able to combine desirable qualities of taste and appearance in tubers or fruits with higher levels of resistance remains to be seen.

Host-selection patterns in the Solanaceae seem to be dominated by the ability of oligophagous insects to overcome allomonal effects, rather than by their requirement of unique kairomones. Solanaceae-associated arthropods seem to be able to respond to host-plant odors, common leaf factors with no apparent allomonal properties (Visser, 1979). Allomonal and kairomonal effects in this system seem to result from different sets of compounds.



Glucosinolates:

Sinigrin



Sinalbin

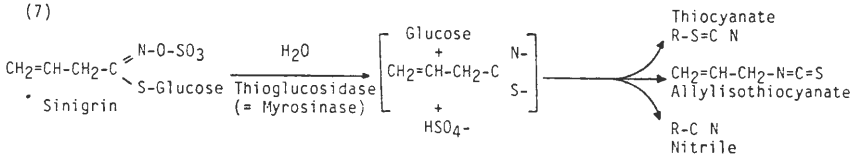
DEFENSIVE PATTERNS IN THE CRUCIFERAE

Verschaffelt's (1910) paper on the relationship of cabbage butterflies (*Pieris* spp.) with crucifers pioneered modern concepts in insect/plant interactions and coevolution. This system provided the first unmistakable evidence of the allelochemical role of secondary plant metabolites. Many crucifers are preferred vegetable foods in human diet. The chemistry of glucosinolates and their associated isothiocyanates, the odor and taste factors in crucifers, has been studied in detail since the 1830s. The structural formulas for sinigrin and sinalbin (6), two of the most common "mustard-oil" glycosides

(glucosinolates), were determined in the mid 1890s (see review by Underhill, 1980).

Glucosinolates are hydrolyzed by the enzyme myrosinase when the plant tissue is crushed. The products of hydrolysis are glucose, sulfate, and an aglucone that may undergo intramolecular rearrangement and produce an isothiocyanate (7).

(7)



Glucosinolates occur in practically all species studied in five closely related families, including the Cruciferae, Capparidaceae, and Resedaceae, and in several species in six other unrelated families, including the Tropaeolaceae and Euphorbiaceae (Underhill, 1980). There are about 80 different kinds of glucosinolates and major groups may be characteristic of certain families. They typically occur as mixtures in each plant species (see Van Etten, 1969; Van Etten and Tookey, 1979).

Glucosinolates or their breakdown products are potent allomones. They are well known for their goitrogenous effects on humans and other vertebrates (Van Etten, 1969). When fed to noncrucifer associated insects, glucosinolates have a marked antibiotic effect. *Papilio polyxenes* Fabr. larvae, oligophagous on Umbelliferae; *Spodoptera eridania* (Cramer), a polyphagous species; and *Pieris rapae* L., oligophagous on crucifers, were fed acceptable plants (carrot, lima bean, and collards, respectively) that were cultured in solutions containing various concentrations of allylglucosinolate. Absorption of the compound by the plant cutting was confirmed by gas chromatography. The result was a sharp decline in growth efficiency (regression of relative growth rate on relative consumption rate) as glucosinolate concentration increased for *P. polyxenes*, no effect for *P. rapae*, and an intermediate effect for *S. eridania* (Blau et al., 1978). Thus, glucosinolates have an acute effect for oligophagous insects not associated with crucifers and a moderate dose-related effect for polyphagous species (Lerin, 1980).

The striking feature of glucosinolates is their potent attraction and feeding-excitation for species associated with crucifers. Attraction of adults or larvae of insects in five orders (Lepidoptera, Coleoptera, Diptera, Homoptera, and Hymenoptera) has been demonstrated by

field and laboratory experiments. Adults of these species are attracted and stimulated to oviposit by specific glucosinolates of the host plant. The same compounds attract larvae and elicit feeding responses. The mix and relative concentrations of glucosinolates vary with host species and environmental conditions. Consequently, the patterns of host exploitation are also variable. Host ranges of crucifer phytophages are apparently influenced by the glucosinolate profile of the plant (see Rodman and Chew, 1980 and references therein).

Although glucosinolates have an important role in the Cruciferae/arthropod associations, they do not entirely explain the observed patterns of host specificity. Nielsen et al., (1979) reported that the horseradish flea beetle, *Phyllotreta armoraciae* (Koch), is restricted to feeding on horseradish, as it responds specifically to a combination of allylglucosinolate and two flavonol glycosides: kaempferol-3-0-xylosylgalactoside and quercitin 3-0-xylosylgalactoside. The kaempferol glycoside is the most active; *P. armoraciae* does not recognize horseradish in its absence.

Some wild crucifers contain other potent allomones and are less susceptible to flea beetle species normally associated with crucifers. Cucurbitacins E and I were reported from green parts and seeds of *Iberis*, and cardenolides occur in species of *Cheirantus* and *Erysimum* (Nielsen, 1978, and references therein). Several species of the genus *Phaedon* (Coleoptera: Chrysomelidae) were offered either horseradish leaf discs treated with pure cucurbitacins or cardenolides and leaf discs of the plants naturally containing those compounds. In all cases, flea beetles displayed reduced acceptance of both the foliage containing those allomones and foliage treated with them. Nielsen (1978) suggested that cucurbitacins and cardenolides represent a second level of chemical defense in the Cruciferae.

In summary, the ubiquitous glucosinolates seem to perform multiple roles in this system. They are not only equally active in host acceptance for oviposition and for feeding but are also powerful allomones for noncrucifer feeders. Other volatiles may be required for attraction to specific hosts, and flavonol glycosides are essential for the oligophagous relationship of *Phyllotreta armoraciae* with horseradish. Several cruciferous genera contain cucurbitacins or cardenolides in addition to glucosinolates, and they are less susceptible to certain flea beetles.

The implications to host-plant resistance are many. Glucosinolates have a major role in the flavor of cruciferous vegetables for both humans and insect pests. Breeding for reduced or zero glucosinolates, if desirable or possible, would expose plants to attacks by potential

pests presently deterred by the allomonal properties of glucosinolates. Reduced glucosinolates would certainly change the crucifer quality as human food. Breeding for high glucosinolate content, on the other hand, may increase resistance but alter flavor and increase the risk of goitrogeny. Manipulation of the odd, second level metabolites, cucurbitacins and cardenolides, has not been attempted, as the genera in which they occur are taxonomically and, probably, genetically distant from the cultivated species.

DEFENSIVE PATTERNS IN THE GRAIN LEGUMES

The Leguminosae are a large, chemically diverse family (Harborne et al., 1971); therefore, only plants in the *Phaseoleae*, mainly grain legumes in the genera *Glycine* and *Phaseolus*, are discussed here. By contrast with the Solanaceae and Cruciferae, grain legumes lack dominant secondary metabolites with obvious allelochemic potential. A cursory list of phytochemicals found in species of *Glycine* and *Phaseolus* is shown in Table 2 (based mainly on Harborne et al., 1971).

The cultivated species of grain legumes harbor a rich arthropod fauna. Most of the North American species associated with *Phaseolus*, a genus with many native Nearctic representatives, have adapted well to soybean, a relatively recent introduction into the region (Kogan, 1981). However, comparisons of host preferences of one of the oligophagous *Phaseolus* phytophages, the Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant, show that preferences followed a certain order: *P. vulgaris* = *P. lunatus* > susceptible soybean lines > cv. Harosoy = Clark = Bragg > *P. aureus* = *P. mungo* > soybean PI 229358 >> PI 171451 = 227687 (Kogan, 1972). PI's 171451, 227687, and 229358 have high levels of resistance to the MBB (Van Duyn et al., 1971). Resistance in these lines has been later confirmed to hold also against several lepidopterous larvae, the bean leaf beetle (*Cerotoma trifurcata* Forster), blister beetles, stink bugs (in the laboratory) (Clark et al., 1972), and even the adult of an Australian stem borer, *Zygrita diva* Thomson (Coleoptera: Cerambycidae) (Shepard et al., 1981).

Although expression of resistance in these lines is consistently demonstrated in field and laboratory, the search for the chemical resistance factors has been frustrating. The difficulty probably lies in the nature of the host selection mechanisms of grain legume-associated arthropods. Most research in this area has been done with the MBB, which is one of the standard experimental insects in our laboratory.

Table 2. Some characteristic secondary metabolites found in various plant parts of *Phaseolus* spp. and *Glycine* spp. (based mainly on Harborne et al., 1971).

Secondary Plant Compound	*Phaseolus*	*Glycine*
Flavonoids		
Flavonols/ones	Robinin	-
	Quercitin-3-glucuronide	-
Isoflavones	Daidzein	Daidzein
		Genistein
		Glyceolins
		Coumestrol
		Sojagol
Alkaloids	Guanidine	-
	Trigonelline	-
Carbohydrates	Raffinose	Raffinose
	Stachyose	Stachyose
	Myoinositol	
Steroids	Stigmasterol	Stigmasterol[b]
	α Sitosterol	α Sitosterol
		Campesterol
	Soyasapogenol-C (glycoside)	Soyasapogenols A-E (glycosides)
	Phaseolin	-
Lipids	-	Ceramides[a]
Miscellaneous	Linamarin	Cerebrosides[a]
	Lotaustralin	-
Phenolic acids		Gallic[c]
		Protocatechuic
		p-Hydroxybenzoic
		Vanillic
		Caffeic
		p- Coumaric
		Ferulic

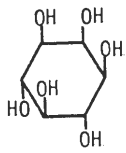
[a]Ohnishi et al., (1982)
[b]Grunwald and Kogan (1981)
[c]Hardin (1979)

Early searches for feeding excitants for the MBB suggested that cyanogenic glycosides extracted from leaves of *Phaseolus vulgaris* and *P. lunatus*, particularly linamarin and lotaustrin, elicited biting response in 3rd instar MBB larvae when a 0.15M glucose solution was also present (Nayar and Faenkel, 1963). Filter paper or lettuce discs, both neutral substrates, treated with solutions of these glycosides were scratched by the beetle attempting to feed. The concentration of nonreducing sugars also has been proposed as an important factor in host discrimination for the MBB among nine species of grain legumes (Lapidus et al., 1963).

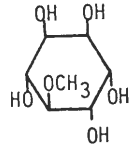
The MBB is a peculiar and fastidious feeder. Because of its unique feeding method, it is sensitive to both the chemical nature and physical consistency of the diet. The nature of this required physical substrate is still unclear. Recent experiments have shown that close-range chemoreceptors in the antennae and mainly in the maxillary palps are responsible for host discrimination. Ablation of these appendages causes the beetle to lose its ability to discriminate between susceptible and resistant soybean lines (Fischer and Kogan, 1986). As there seems to be no biting response until the beetle receives the proper olfactory signal, we are now concentrating on the low molecular weight volatiles of *P. vulgaris* and susceptible and resistant soybean lines. It seems that four or five simple organic compounds and probably one inorganic gas are at the root of behavioral responses in the early stages of host selection (Wei, Kogan and Fischer, Univ. of Illinois, unpublished data).

Attempts to elucidate the chemical bases of resistance in PI's 171451, 227687, and 229358 have revealed the following:

1. Foliar total nitrogen, soluble carbohydrates, organic acids and sterols of both resistant and susceptible soybean vary with stage of growth (Tester, 1977; Grunwald and Kogan, 1981).
2. Total nitrogen was generally lower and, at the end of the season, soluble carbohydrates and sterols were higher in the resistant lines; organic acids fluctuated without correlation with resistance (Tester, 1977).
3. Sterol profiles were similar in both susceptible and resistant lines, both in early and in late maturing soybeans (Grunwald and Kogan, 1981).
4. Pinitol (8) is a dominant cyclitol in soybean and it represents an average of 60% of the total 80%-ethanol-soluble carbohydrate fraction of soybean (Phillips et al., 1982).



myo-Inositol



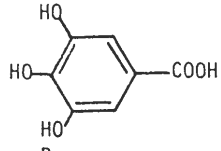
(8)

(+)-Pinitol

5. Pinitol was found in large amounts in PI 229358, and in smaller concentrations in the susceptible cultivar 'Davis' (@ 1% dry weight). Added to artificial media at 0.8%, pinitol reduced weight gain by corn earworm larvae by about 63%. These findings suggest that pinitol is an antibiotic factor in soybean but with no apparent antixenotic property (Dreyer et al., 1979). As the reported results could not be replicated elsewhere, this claim has been recently disputed (Gardner et al., 1984).

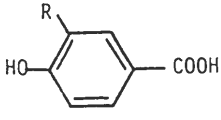
6. Systematic fractionation of PI's 227687 (Smith and Fischer, 1983) and 229358 (Binder and Waiss, 1984) yielded fractions with intermediate and with polar solvents that caused slower developmental rates and high mortality in soybean looper [*Pseudoplusia includens* (Walker)] and *Heliothis zea* Boddie, respectively.

7. Seven phenolic acids were detected in PI 171451 and in cv. 'Forrest': gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic,

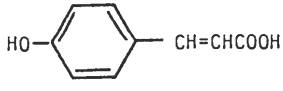


(9)

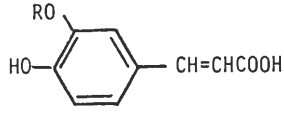
Gallic acid



R= H, p-Hydroxybenzoic Acid
R=OH, Protocatechuic Acid
R=OMe,Vanillic Acid



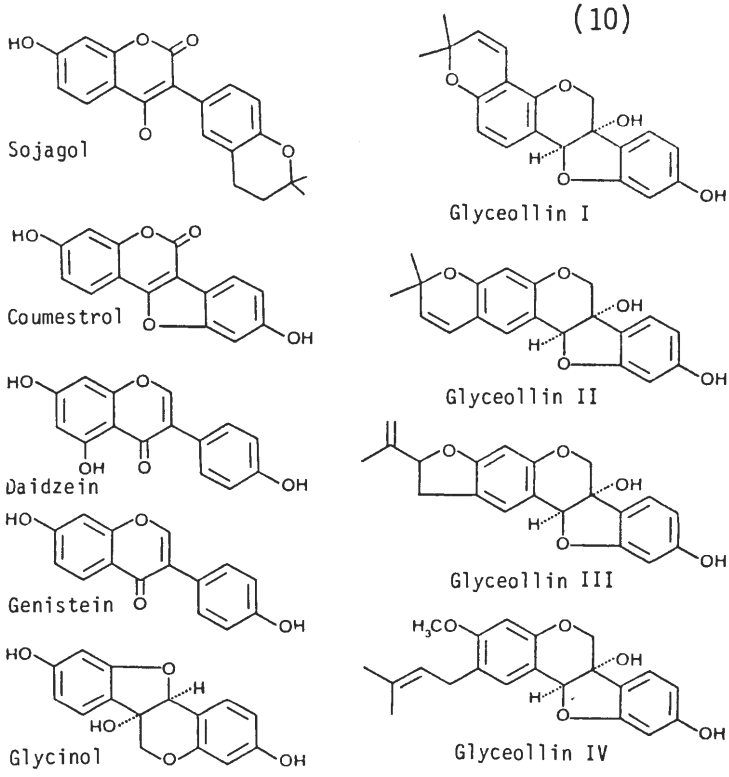
p-Coumaric Acid



R= H, Caffeic Acid
R=Me, Ferulic Acid

p-coumaric, and ferulic (9). The potentially antibiotic caffeic and ferulic acids occurred at higher concentrations in the resistant PI than in the susceptible cultivar. In both types of plants, concentrations were higher in injured than in uninjured tissue (Hardin, 1979).

8. Isoflavonoid phytoalexins (10) in soybean cotyledons are potent feeding deterrents for the Mexican bean beetle (Hart et al., 1983). These phytoalexins are biochemically related to the phenolics mentioned above, through the shikimic acid pathway.



Resistance in soybean to the MBB operates both at the pre-ingestion (antixenosis) and the post-ingestion phases of the host-selection process. Recent work in our laboratory suggests that *Phaseolus* may have a mild attractant that *Glycine* lacks. Consequently,

the olfactory component in resistance is probably related to mild feeding deterrents or to some subtle close range repellent in addition to lack of attractants. This assumption is based on an extensive series of dual-choice tests and on the results of ablation experiments (see above).

As feeding and ingestion proceed, two groups of factors are activated. First, the insect ingests a diet that may be imbalanced in nitrogen/carbon proportions; the prevalent simple carbohydrate, pinitol, may have some mild detrimental effects; and yet unknown constitutive phytochemicals (saponins, glycosides, or some others) may affect normal digestion and assimilation (Kogan, 1972). Second, the injured tissue activates a stress response system that induces phytoalexin production. As phytoalexin metabolism is related to the shikimic-polymalonic acid pathway (Stoessl, 1982), the typical phenolic acids of this pathway become more abundant and increase the antibiotic reaction of the plant. Both susceptible and resistant soybean genotypes would have the same basic biochemical mechanisms, but they would differ in the response rate. *Phaseolus* spp., however, would lack parts of this defensive scheme; hence, it is a better host for the MBB than either susceptible or resistant soybeans.

This scenario is speculative. Some crucial experimental steps are still missing and the phytochemical profiles of resistant and susceptible soybeans and common beans are far from complete.

CONCLUSIONS

Breeding for insect resistant cultivars has advanced for many years without full knowledge of the underlying biochemical mechanisms. Programs have been generally slow and erratic. Mechanisms of resistance to insects are seldom simple, and few, if any, systems have been fully elucidated. Future generations of genetic engineers will probably demand insight into the intricacies of these biochemical mechanisms, their enzymatic mediation, their genetic regulation, and their environmental modulation. However, rather than attempting to justify the need for this knowledge on some promise of uncertain future achievements, I believe that the full potential of the use of host plant resistance in pest control will be realized when we begin to understand insect/plant interactions in a broad ecological context.

Chemical defenses represent but one facet of the complex strategies that plants have evolved to resist herbivore attacks. Other components of these strategies are temporal and spatial escape, compensatory growth (= tolerance), arrays of physical defenses, and

interactions with other mortality factors that regulate populations of the plant's associated herbivores (parasitoids, predators and diseases). Interactions among all these factors form the ecological context of the crop plant and its associated fauna. To fully understand the role of secondary metabolites in host plant resistance, one should consider all elements and their complex interactions within this ecological context.

Secondary plant metabolites may enhance or attenuate the actions of other mortality factors. Some metabolites may be used by herbivores for their own protection against predators (Duffey, 1980). Evaluation of the possible interactions is essential for understanding the role of secondary metabolites in insect/plant interactions and in host-plant resistance.

The three systems discussed above represent different models of plant defense strategy. In the Solanaceae, allomonal and kairomonal functions seem to rest with very diverse groups of compounds. Polyphenols and keto-hydrocarbons in cuticular trichomes and the steroidal and other alkaloids are antibiotic and antixenotic factors. Some of the alkaloids may be tolerated, neutralized, or even sequestered for their own defenses by Solanaceae-oligophagous herbivores. Host recognition and acceptance, however, are elicited by compounds with strictly kairomonal functions.

In the Cruciferae, glucosinolates apparently act as both allomones and kairomones, even for crucifer-oligophages. Here, qualitative and quantitative differences in glucosinolate profiles determine the pattern of host recognition, acceptance, and suitability. Certain flavonol glycosides may also be essential for host specificity in some species. An additional line of defense occurs in a few genera that produce cucurbitacins and cardenolides.

The Leguminosae apparently have no overpowering allomones. Instead, a complex system based on the absence of strong attractants and the presence of mild repellents, nutritionally imbalancing factors, and injury-induced phenolics, combine to resist the threatening fauna.

This knowledge has not been fully appreciated in practical breeding programs. Possible applications in screening by chemical-analytical means are often cited but seldom realized because of the cost and time involved. Major benefits will come from the capability to develop and evaluate breeding stategies based on detailed information of the plants' chemical defenses, their metabolic origin, and genetic regulation. The rationale for this proposition is diagrammed in Figure 3 and is based on the following reasoning. If a plant has a limited pool

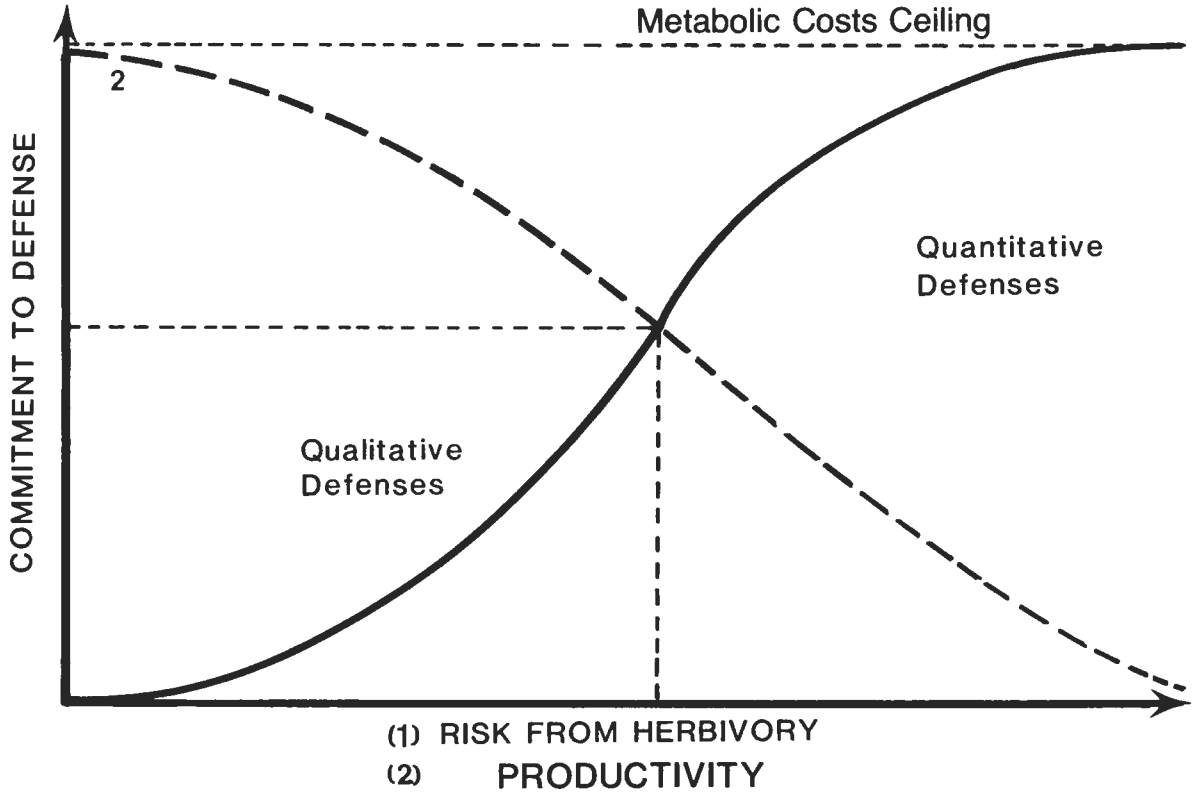


Figure 3. Theoretical relationship between a plant's energetic investment in the biosynthesis of defense phytochemicals and productivity (= yield). If commitment to defense is virtually nihil, productivity would reach a maximum only in the absence of pests. However, plants may have evolved defensive phytochemicals in proportion to risk from attacks; the energy invested would approach a metabolic cost ceiling above which there is no reproduction and the plant (and genes for such defenses) is eliminated.

of metabolizable energy to perform its vital functions (growth, reproduction, competition, and defense), then each incremental demand for one function will be met at the expense of the others. Chemical defense must have a certain metabolic cost that may impinge on the energy resources needed for maximum yield. Aiming at ever higher yield levels may drain energy needed for defense and plants will become more vulnerable. By understanding the chemical bases of resistance we may avoid the frustration of pursuing unattainable goals. Conversely, that understanding may help us set more realistic goals in reaching a compromise between maximum yields coupled with a reasonable defense capability.

A breeding strategy attempting to integrate all these factors will require a good team effort. When we reach this level of integration, breeding for resistance will be a true science, no longer an art.

ACKNOWLEDGMENT

I thank Daniel Fischer, Charles Helm (INHS), and Don Strong (Florida State University) for helpful comments. This publication is a contribution of the Illinois Natural History Survey and Illinois Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign, supported in part by Hatch Project ILLU-12-0324, the Regional Project S-157, and by a USDA grant 82-CRSR-2-1000, through the Consortium for Integrated Pest Management and Texas A&M University. The opinions expressed herein are those of the author and not necessarily those of the supporting institutions or agencies.

LITERATURE CITED

Beck, S. D. 1965. Resistance of plants to insects. Annu. Rev. Entomol. 10:207-232.

Bell, E. A. and B. V. Charlwood. 1980. Secondary plant products. 674 pp. Springer-Verlag, Berlin.

Binder, R. G. and A. C. Waiss, Jr. 1984. Effects of soybean leaf extracts on growth and mortality of bollworm (Lepidoptera: Noctuidae) larvae. J. Econ. Entomol. 77:1585-1588.

Bisby, F. A., J. G. Vaughan, and C. A. Wright (eds.). 1980. Chemosystematics: Principles and practice. 449 pp. Academic Press, London.

Blau, P. A., P. Feeny, L. Contardo, and D. S. Robson. 1978. Allylglucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. Science 200:1296-1298.

Bordner, J. and collaborators. 1983. Chemical bases for host plant selection, pp. 245-264. *In*: P. Hedin (ed.). Plant resistance to insects. ACS Symposium Ser. 208, American Chemical Society, Washington, DC.

Broadway, R. M., S. S. Duffey, G. Pearie, and C. A. Ryan. (in press). Plant proteinase inhibitors: a defense against herbivores? Entomol. Exp. Appl.

Chapman, R. F. and E. A. Bernays. 1977. The chemical resistance of plants to insect attack, pp. 603-643. *In*: G. B. Marini-Bettolo (ed.). Natural products and the protection of plants. Pontificia Academiae Scientiarum Scripta Varia 41, Vatican.

Clark, W. J., F. A. Harris, F. G. Maxwell, and E. E. Hartwig. 1972. Resistance of certain soybean cultivars to bean leaf beetle, striped blister beetle, and bollworm. J. Econ. Entomol. 65:1669-1672.

Crawley, M. J. 1983. Herbivory: The dynamics of animal-plant interactions. 437 pp. Univ. of California Press, Berkeley.

Dethier, V. G. 1954. Evolution of feeding preferences in phytophagous insects. Evolution 8:33-54.

————. 1970. Chemical interactions between plants and insects, pp. 83-102. *In*: E. Sondheimer and J. B. Simeone (eds.). Chemical ecology. Academic Press, New York.

————, L. Barton Brown, and C. N. Smith. 1960. The designation of chemicals in terms of the responses they elicit from insects. J. Econ. Entomol. 53:134-136.

Dreyer, D. L. and collaborators. 1979. Pinitol, a larval growth inhibitor for *Heliothis zea* in soybeans. Experientia 35:1182-1183.

Duffey, S. S. 1980. Sequestration of plant natural products by insects. Annu. Rev. Entomol. 25:447-477.

Ehrlich, P. R. and P. H. Raven. 1964. Butterflies and plants: a study in coevolution. Evolution 18:586-608.

F.A.O. 1981. 1980 FAO production yearbook. Vol. 34. Food and Agriculture Organization of the United Nations. 296 pp. Rome.

Fischer, D. and M. Kogan. 1986. Chemoreceptors of adult Mexican bean beetles: Structure and role in food preference. Entomol. Exp. Appl. 40:3-12.

Fraenkel, G. 1959. The *raison d'etre* of secondary plant substances. Science 129:1466-1470.

————. 1969. Evaluation of our thoughts on secondary plant substances. Entomol. Exp. Appl. 12:473-486.

Futuyma, D. J. 1983. Evolutionary interactions among herbivorous insects and plants, pp. 207-231. *In*: D. J. Futuyma and M. Slatkin (eds.). Coevolution. Sinauer Assoc. 555 pp. Sunderland, MA.

———— and M. Slatkin (eds.). 1983. Coevolution. Sinauer Assoc. 555 pp. Sunderland, MA.

Gardner, W. A., D. V. Phillips, and A. E. Smith. 1984. Effect of pinitol on the growth of *Heliothis zea* and *Trichoplusia ni* larvae. J. Agr. Entomol. 1:101-105.

Gibson, R. W. 1971. Glandular hairs providing resistance to aphids in certain wild potato species. Ann. Appl. Biol. 68:113-119.

Gregory, P. 1984. Glycoalkaloid composition of potatoes: Diversity and biological implications. Amer. Potato J. 61:115-122.

Grunwald, C. and M. Kogan. 1981. Sterols in soybean differing in insect resistance and maturity group. Phytochemistry 20:765-768.

Harborne, J. B. 1982. Introduction of ecological biochemistry. 278 pp. Academic Press, London.

————, D. Boulter, and B. L. Turner. 1971. Chemotaxonomy of the Leguminosae. 612 pp. Academic Press, London.

Hardin, J. M. T. 1979. Phenolic acids of soybeans resistant and nonresistant to leaf feeding larvae. 50 pp. M.S. Thesis, University of Arkansas.

Harlan, J. R. 1975. Crops and man. 295 pp. Amer. Soc. Agronomy, Madison, WI.

Harris, M. K. (ed.). 1980. Biology and breeding for resistance to arthropods and pathogens in agricultural plants. 605 pp. Texas Ag. Exp. Sta. MP - 1451, Texas A & M University, College Station, TX.

Hart, S. V., M. Kogan, and J. D. Paxton. 1983. Effect of soybean phytoalexins on the herbivorous insects Mexican bean beetle and soybean looper. J. Chem. Ecol. 9:657-672.

Hedin, P. A. 1977. Host plant resistance to pests. 286 pp. A.C.S. Symposium Ser. 62, American Chemical Society, Washington, DC.

————. 1983. Plant resistance to insects. 375 pp. A.C.S. Symposium Ser. 208, American Chemical Society, Washington, DC.

————, J. N. Jenkins, and F. G. Maxwell. 1977. Behavioral and developmental factors affecting host plant resistance to insects, pp. 231-275. *In*: P. A. Hedin (ed.). Host plant resistance to pests. 286 pp. A.C.S. Symp. Ser. 62, American Chemical Society, Washington, DC.

Horber, E. 1980. Types and classification of resistance, pp. 15-21. *In*: F. G. Maxwell and P. A. Jennings (eds.). Breeding plants resistant to insects. 683 pp. John Wiley, New York.

Hsiao, T. H. and G. Fraenkel. 1968a. The role of secondary plant substances in the food specificity of the Colorado potato beetle. Ann. Entomol. Soc. Amer. 61:485-493.

———— and ————. 1968b. Selection and specificity of the Colorado potato beetle for solanaceous and nonsolanaceous plants. Ann. Entomol. Soc. Amer. 61:493-503.

Isman, M. B. and S. S. Duffey. 1980. Toxicity of tomato phenolic compounds to the fruitworm, *Heliothis zea*. Entomol. Exp. Appl. 31:370-376.

Janzen, D. 1980. When is it coevolution? Evolution 34:611-612.

Jermy, T. 1966. Feeding inhibitors and food preferences in chewing phytophagous insects. Entomol. Exp. Appl. 9:1-12.

Kogan, M. 1972. Feeding and nutrition of insects associated with soybeans. 2. Soybean resistance and host preferences of the Mexican bean beetle, *Epilachna varivestis*. Ann. Entomol. Soc. Amer. 65:675-683.

———. 1977. The role of chemical factors in insect/plant relationships, pp. 122-227. *In*: Proc. 15th Int. Congr. Entomol., Washington, DC.

———. 1980. Insect problems of soybean in the United States, pp. 303-325. *In*: F. T. Corbin (ed.). World Soybean Research Conference II: Proceedings. Raleigh, North Carolina. 857 pp. Westview Press, Boulder, CO.

———. 1981. Dynamics of insect adaptations to soybean: Impact of integrated pest management. Environ. Entomol. 10:363-371.

———. 1982. Plant resistance in pest management, pp. 93-134. *In*: R. L. Metcalf and W. H. Luckmann (eds.). Introduction to insect pest management. 577 pp. 2nd ed., John Wiley, New York.

——— and R. D. Goeden. 1971. Feeding and host-selection behavior of *Lema trilineata daturaphila* larvae (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Amer. 64:1435-1448.

——— and E. E. Ortman. 1978. Antixenosis—A new term proposed to define Painter's "nonpreference" modality of resistance. Bull. Entomol. Soc. Amer. 24:175-176.

Lapidus, G. S. and collaborators. 1963. Chemical factors influencing host selection in the Mexican bean beetle *Epilachna varivestis* Muls. J. Agric. Food Chem. 11:462-463.

Lerin, J. 1980. Influence des substances allelochimiques des cruciferes sur les insectes. Acta Oecol. 1:215-235.

Maxwell, F. G. 1977. Host plant resistance to insects—Chemical relationships, pp. 299-304. *In*: H. H. Shorey and J. J. McKelvey, Jr. (eds.). Chemical control of insect behavior: Theory and application. 414 pp. John Wiley, New York.

Mothes, K. 1980. Historical introduction, pp. 1-10. *In*: E. A. Bell and B. V. Charlwood (eds.). Secondary plant products. Encyclopedia of plant physiology, vol. 8. 674 pp. Springer-Verlag, Berlin-Heidelker.

Nayar, J. K. and G. Fraenkel. 1963. The chemical basis of host selection in the Mexican bean beetle, *Epilachna varivestis* (Coleoptera, Coccinellidae). Ann. Entomol. Soc. Amer. 56:174-178.

Nelson, C. E. and collaborators. 1983. Regulation of synthesis and accumulation of proteinase inhibitors in leaves of wounded tomato plants, pp. 103-122. *In*: P. A. Hedin (ed.). Plant resistance to insects. ACS Symposium Ser. 208. 375 pp. American Chemical Society, Washington, DC.

Nielson, J. K. 1978. Host plant discrimination within Cruciferae: Feeding responses of four leaf beetles (Coleoptera: Chrysomelidae) to glucosinolates, cucurbitacins and cardenolides. Entomol. Exp. Appl. 24:41-54.

———, L. M. Larsen, and H. Sorensen. 1979. Host plant selection of the horseradish flea beetle *Phyllotreta armoraciae* (Coleoptera: Chrysomelidae):

Identification of two flavonol glycosides stimulating feeding in combination with glucosinolates. Entomol. Exp. Appl. 26:40-48.

Norris, D. M. and M. Kogan. 1980. Biochemical and morphological bases of resistance, pp. 23-61. *In*: F. G. Maxwell and P. R. Jennings (eds.). Breeding plants resistance to insects. 683 pp. John Wiley, New York.

Ohnishi, M., S. Ito, and Y. Fujino. 1982. Chemical characterization of ceramide and cerebroside in soybean leaves. Agric. Biol. Chem. 46:2855-2856.

Painter, R. H. 1951. Insect resistance in crop plants. 520 pp. Macmillian Co., New York.

Phillips, D. V., D. E. Dougherty, and A. E. Smith. 1982. Cyclitols in soybean. J. Agric. Food Chem. 30:456-458.

Rodman, J. E. and F. S. Chew. 1980. Phytochemical correlates of herbivory in a community of native and naturalized Cruciferae. Biochem. Syst. Ecol. 8:43-50.

Rosenthal, G. A. and D. H. Janzen. 1979. Herbivores: Their relation with secondary plant metabolites. 718 pp. Academic Press, New York.

Ryan, C. A. 1973. Proteolytic enzymes and their inhibitors in plants. Annu. Rev. Plant Physiol. 24:173-196.

————. 1979. Proteinase inhibitors, pp. 599-618. *In*: G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their interaction with secondary plant metabolites. 718 pp. Academic Press, New York.

Ryan, J. D., P. Gregory, and W. M. Tingey. 1983. Glandular trichomes: Enzymic browning assays for improved selection of resistance to the green peach aphid. Amer. Potato J. 60:861-868.

Shepard, M., M. Keerati-kasikorn, P. R. B. Blood, and D. Morgan. 1981. Soybean resistance to adults of lucerne crown borer, *Zygrita diva* Thomson (Coleoptera: Cerambycidae). J. Ga. Entomol. Soc. 16:34-40.

Smith, C. M. and N. H. Fischer. 1983. Chemical factors of an insect resistant soybean genotype affecting growth and survival of the soybean looper. Entomol. Exp. Appl. 33:343-345.

Stoessl, A. 1982. Biosynthesis of phytoalexins, pp. 133-180. *In*: J. A. Bailey and J. W. Mansfield (eds.). Phytoalexins. 334 pp. John Wiley, New York.

Strong, D. R., J. H. Lawton, and R. Southwood. 1984. Insects on plants: Community patterns and mechanisms. 313 pp. Harvard University Press, Cambridge, MA.

Tester, C. F. 1977. Constituents of soybean cultivars differing in insect resistance. Phytochemistry 16:1899-1901.

Thorsteinson, A. J. 1960. Host selection of phytophagous insects. Annu. Rev. Entomol. 5:193-218.

Tingey, W. M. and R. W. Gibson. 1978. Feeding and mobility of the potato leafhopper impaired by glandular trichomes of *Solanum berthaultii* and *S. polyadenium*. J. Econ. Entomol. 71:856-858.

———— and S. L. Sinden. 1982. Glandular pubescence, glycoalkaloid composition, and resistance to the green peach aphid, potato leafhopper, and potato flea beetle in *Solanum berthaultii*. Amer. Potato J. 59:95-105.

Toldine, T. E. 1984. Relationship between DIMBOA content and *Helminthosporium turcicum* resistance in maize. (In Hungarian with English abstract.) Novenytermeles 33:213-218.

Underhill, E. W. 1980. Glucosinolates, pp. 493-511. *In*: E. A. Bell and B. V. Charlwood (eds.). Secondary plant products. 674 pp. Springer-Verlag, Berlin.

Van Duyn, J. W., S. G. Turnipseed, and J. D. Maxwell. 1971. Resistance in soybeans to the Mexican bean beetle. I. Sources of resistance. Crop Sci. 11:572-573.

Van Etten, C. H. 1969. Goitrogens, pp. 103-142. *In*: I. E. Liener (ed.). Toxic constituents of plant foodstuffs. 500 pp. Academic Press, New York.

———— and H. L. Tookey. 1979. Chemistry and biological effects of glucosinolates, pp. 471-500. *In*: G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their interaction with secondary plant metabolites. 718 pp. Academic Press, New York.

Verschaffelt, E. 1910. The cause determining the selection of food in some herbivorous insects. Proc. Royal Acad. Amsterdam. 13:536-542.

Visser, J. H. 1979. Olfaction in the Colorado potato beetle at the onset of host plant selection. PUDOC. 88 pp. Wageningen Press, Holland.

Whittaker, R. H. and P. P. Feeny. 1971. Allelochemics: Chemical interactions between species. Science 171:757-770.

Williams, W. G. and collaborators. 1980. 2-Tridodecanone: A naturally occurring insecticide from the wild tomato *Lycopercicon hirsutum* F. *glabratum*. Science 207:888-889.

Yamamoto, R. T. and G. S. Fraenkel. 1960. The specificity of the tobacco hornworm, *Protoparce sexta*, to solanaceous plants. Ann. Entomol. Soc. Amer. 53:503-507.

IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 529-543
Vol. 60, No. 4

METABOLIC REGULATION IN PLANT-PATHOGEN INTERACTIONS FROM THE PERSPECTIVE OF THE PATHOGEN

Tsune Kosuge[1]

ABSTRACT. The relationship between metabolic regulation, virulence, and fitness in plant pathogens is discussed with indoleacetic acid (IAA) production by *Pseudomonas savastanoi* as a model. Production of IAA confers virulence in the bacterium for its hosts, oleander and olive. The bacterium produces IAA from tryptophan by the sequence of reactions L-tryptophan ⟶ indoleacetamide ⟶ indoleacetic acid. The enzymes concerned and their genetic determinants are: tryptophan 2-monooxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*). The two genes, *iaaM* and *iaaH*, which are virulence genes for the bacterium, occur on a plasmid, pIAA, in oleander strains and on the chromosome of olive strains of the pathogen. The virulence gene product, tryptophan monooxygenase, diverts tryptophan into the production of IAA; thus, the enzyme not only helps confer virulence but also regulates the production of IAA and the diversion of the primary metabolite, L-tryptophan, into secondary metabolism. The enzyme is a flavoprotein and is product inhibited by indoleacetamide (K_i=7 μM); the inhibition is competitive with respect to the substrate, L-tryptophan. Control of IAA production and virulence therefore occurs at the step catalyzed by tryptophan monooxygenase.

Two IS-elements, IS-51 and IS-52, resident in the bacterium have been isolated and sequenced. By integration into *iaaM*, they are responsible for loss of IAA production and virulence in several mutants that were isolated by selection for alpha-methyltryptophan resistance. Insertional inactivation of virulence genes by transposable elements similar to IS-51 and IS-52 may be the basis of the spontaneous loss of virulence commonly observed in other plant pathogenic bacteria.

Certain strains of *P. savastanoi* convert IAA to its lysine conjugate; the gene for lysine conjugate synthesis occurs on pIAA, but it is not part of the IAA operon. Conversion of IAA to its lysine conjugate may affect the free pool size of IAA and the amount secreted into host tissue.

Both *iaaM* and *iaaH* have been sequenced; on the basis of both nucleotide and deduced amino acid sequences, the coding sequences of *iaaM* show significant homology with the sequences of the open reading frame of tms-1, one of the two genes in crown gall T-DNA required for IAA production. Less, but nevertheless significant, homology occurs between the coding sequences of *iaaH* and the open reading frame of tms-2 of crown gall T-DNA, the other gene required for IAA production. The results suggest that the genes for IAA production in *P. savastanoi* and crown gall T-DNA have a common origin.

Index descriptors: virulence, fitness, *Pseudomonas savastanoi*, indolacetic acid, plant pathogenic bacteria, plant pathogen interactions.

[1]Department of Plant Pathology, University of California, Davis, CA 95616.

INTRODUCTION

Plant pathogens possess unique metabolic systems that allow them to invade and to cause deleterious effects on their hosts. These specialized systems function together with the normal metabolic processes associated with growth of the pathogen in host tissue. Through various metabolic control systems, a pathogen responds to the ever-changing environment encountered in host tissue. The interaction of all these systems defines the organism's *pathogenicity*, a qualitative term which describes the overall capacity of the organism to cause disease in the plant (Dunkle, 1984). *Virulence* is a quantitative component of pathogenicity and concerns the factors that contribute to the formation and severity of the primary symptoms of disease.

Molecular biological approaches have now made it possible to demonstrate that in some cases virulence can be unequivocally associated with the production of a toxic secondary metabolite by the pathogen; in such cases, expression of virulence can be defined in terms of regulation of secondary metabolism. Further, by isolation of appropriate pathogen mutants, it has been possible to show that secondary metabolism is not essential for the viability of the organism, just as loss of virulence is not lethal to the pathogen. With the use of such experimental material, concepts of virulence and its relationship to metabolic control can be revealed.

In this paper, we discuss metabolic regulation in general; then we use indoleacetic acid (IAA) production by a tumor-forming bacterium, *Pseudomonas savastanoi*, to illustrate how metabolic regulation can be related to expression of virulence in a plant pathogenic bacterium. We describe evidence that shows that IAA is a secondary product formed from the primary metabolite L-tryptophan, and that it serves as a virulence factor in the interaction of the bacterium with its hosts. The results of the studies with *P. savastanoi* may be applicable to virulence expression in other plant pathogenic bacteria. The relation between virulence and pathogen fitness is complex.

PRIMARY METABOLISM DETERMINES GROWTH OF PATHOGEN IN HOST TISSUE

Before virulence can be expressed, a plant pathogenic bacterium such as *P. savastanoi* must grow in its host. It is likely that growth would occur through metabolic processes commonly found in both plant pathogenic and saprophytic bacteria; these processes constitute

the primary metabolism of the bacteria and are those concerned with the production of energy and the formation of essential cellular constitutents such as proteins, polysaccharides and nucleic acids (Aharonwitz, 1980; Atkinson, 1969). All are regulated through intricate and responsive mechanisms which allow orderly growth, maintenance and reproduction of the organism. These systems possess characteristics uniquely designed for their cellular functions (Atkinson, 1969, Aharonwitz, 1980; Crawford and Stauffer, 1980).

Besides determining the growth of the pathogen in host tissue, primary metabolism supplies the precursors and energy for synthesis of secondary metabolites that function as virulence factors. Through this relationship, primary metabolism indirectly contributes to pathogenicity; auxotrophy can be a basis for lack of pathogenicity but not avirulence.

The effectiveness of metabolic control mechanisms determines the efficiency of primary metabolism which in turn, helps determine the fitness of the pathogen. Specific mechanisms of metabolic control therefore provide bases for defining fitness of a pathogen.

SECONDARY METABOLISM IS CONCERNED WITH EXPRESSION OF VIRULENCE

It is known that microorganisms produce a variety of biologically active compounds which arise as secondary products of essential metabolites such as glucose, acetate or one of the essential amino acids (Leisinger and Margraff, 1979). Certain secondary products are produced in host tissue by pathogens and have been identified as virulence factors because they disrupt host cellular functions and account for primary symptoms of diseases (Dunkle, 1984). In *Pseudomonas savastanoi*, IAA functions as a virulence factor as determined by tumor formation, a primary symptom produced by its hosts. Since IAA is a by-product of the metabolism of an essential cellular metabolite, an understanding of its biosynthesis will establish the relationship between expression of virulence and metabolism (Fig. 1). As revealed by studies on IAA production, controls of pathways for synthesis of secondary metabolites exist, just as there are controls over primary metabolism. However, controls over secondary metabolism should differ from those associated with primary metabolism. While they should be responsive to the nutrient and energy status of the cell, they should also ensure that the use of primary metabolites for production of secondary metabolites does not occur at the expense of the essential needs of the cell. Thus, in the wild types of the pathogen,

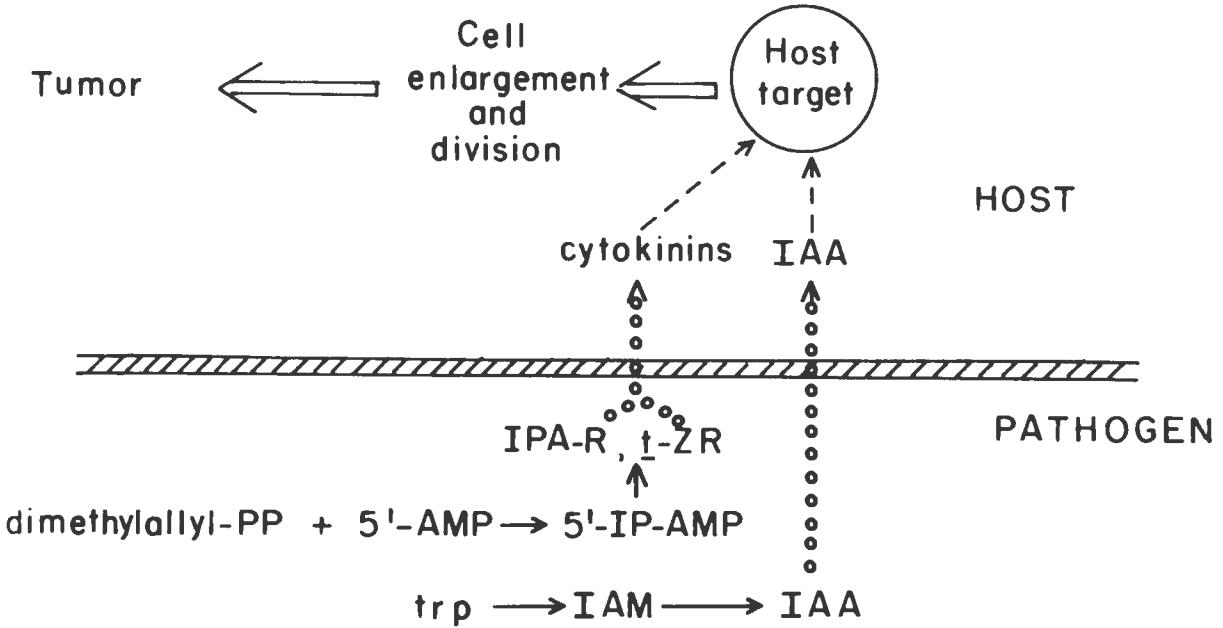


Figure 1. A model for production and secretion by the pathogen of the phytohormones, cytokinins and indoleacetic acid that function as virulence factors in plant-pathogen interactions. The compounds are produced as secondary products by *Pseudomonas savastanoi* and excreted into oleander or olive tissues; the increased concentration of the compounds upsets the normal phytohormone balance in host tissue and causes abnormal proliferation of host cells. The amount of the compounds produced and secreted by the pathogen will directly influence the severity of the symptoms produced by the plant. Therefore, control over the production of the compounds by the pathogen will influence virulence as determined by tumor formation. IAA, indoleacetic acid; IAM, indoleacetamide;trp, tryptophan; IP-AMP, isopentenyl adenine ribotide; IPA-R, isopentenyl riboside; t-ZR, *trans*-zeatin riboside; PP, pyrophosphate.

metabolic controls should be "fine tuned" to assure that metabolism for virulence is appropriately balanced with metabolism concerned with essential life processes. Any alteration in this balanced system could alter virulence at the expense of reduced fitness or competitiveness of the pathogen.

It is commonly observed that virulence varies widely among strains of pathogenic bacteria and fungi and may be lost in any given strain without loss of viability in culture. Such observations are compatible with the notion that virulence factors are products of secondary metabolism or are components of ancillary nonessential metabolic pathways. Moreover, in any given population of a plant pathogen, strains of intermediate virulence occur in the largest numbers while the least and the most virulent strains occur in the smallest numbers. This implies that both hypovirulence and hypervirulence are associated with decreased fitness and decreased capacity to survive in nature. Consequently, if virulence can be associated with the activity of a particular metabolic pathway, it will be possible to determine how the activity of the pathway influences both fitness and expression of virulence. While it is likely that relaxed control over production of a virulence factor relates loss in fitness to hypervirulence, it does not explain the relationship between fitness and hypovirulence. In the latter case, it is likely that the pathway for production of a virulence factor fulfills yet another function in a pathogen and that the activity of the pathway provides some competitive advantage during its existence in nature. Possible relationships between hypovirulence and competitiveness will be discussed later for IAA production in *P. savastanoi.*

INDOLEACETIC ACID PRODUCTION AND VIRULENCE

The association between secondary metabolism and virulence is readily apparent in the interaction between the tumorigenic bacterium, *Pseudomonas savastanoi*, and its hosts, oleander and olive. Production of IAA confers virulence in *P. savastanoi*; mutants deficient in IAA production fail to incite the production of tumors on oleander plants (Fig. 2; Smidt and Kosuge, 1978). When genes for IAA production are transferred from parental strains to these mutants, both IAA synthesis and full virulence are restored (Comai and Kosuge, 1980, 1982). Since loss of IAA production is not lethal to the bacterium, IAA synthesis constitutes secondary metabolism of tryptophan in the bacterium.

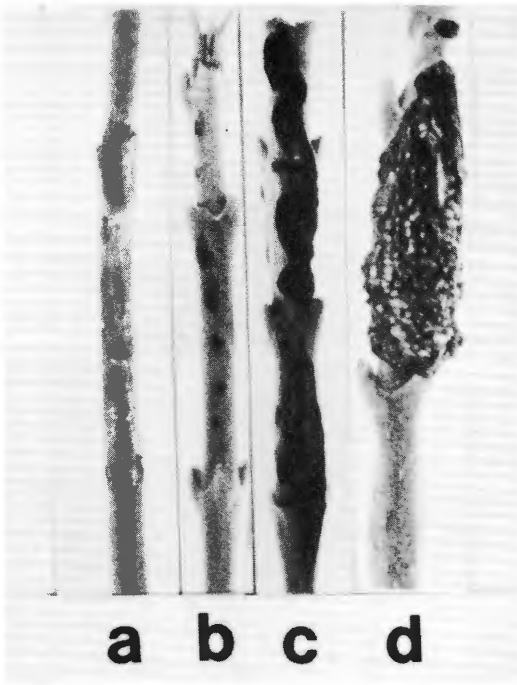


Figure 2. Symptoms produced by oleander plants inoculated with mutant and wild-type strains of *Pseudomonas savastanoi*. A, (control) inoculated with culture medium; B, inoculated with heat-killed cells of the wild type, 2015; C, inoculated with wild type strain, 2015; D, inoculated with the super producer strain, 2015-5. Note the root initials produced on the surface of the tumor tissue.

P. savastanoi produces IAA by the sequence of reactions L-tryptophan ⟶ indoleacetamide ⟶ indoleacetic acid. The enzymes and their genetic determinants are: tryptophan 2-monooxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*) (Comai and Kosuge, 1982, 1983a). These genes (IAA genes) occur on a plasmid, pIAA, in oleander strains and on the chromosome of olive strains (Fig. 3). The plasmid occurs in several sizes ranging from 53 kb (pIAA1) to 72 kb (pIAA2) in different oleander strains (Comai, et al., 1982). Strains can be cured of pIAA readily by selecting for resistance to alpha-methyltryptophan. Such mutants are deficient in IAA synthesis and show loss of virulence. Loss of IAA production can also be attributed

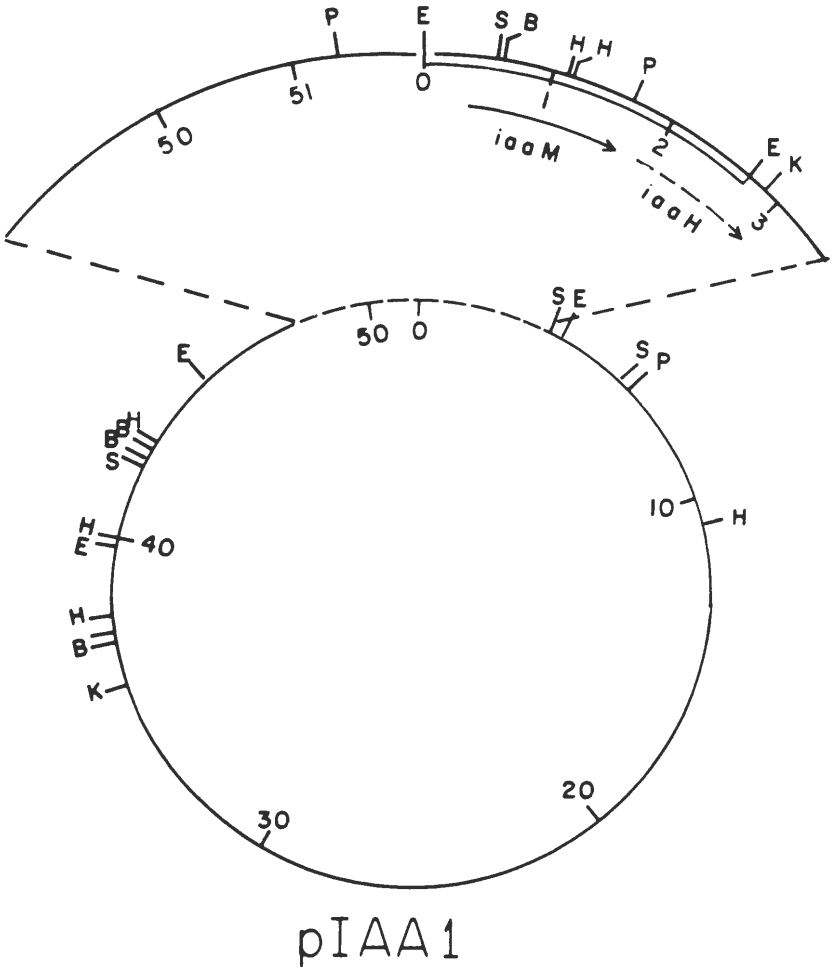


Figure 3. A model of pIAA1. The genetic determinants *iaaM* and *iaaH* probably occur in an operon because insertional inactivation of *iaaM* by IS-elements has polar effects on *iaaH*. The genetic determinant for IAA-lysine synthetase, *iaaL*, occurs upstream from the *iaa* genes and is transcribed in the opposite direction. Restriction endonucleases; P, PST; E, Eco R1; S, Sal I; B, Bam H1; H, Hind III; K, Kpn. Scale is in kilobase pairs.

to deletions of the IAA genes and inactivation of *iaaM* by IS elements such as IS-51 (Comai and Kosuge, 1983b) and IS-52 (Yamada and Kosuge, unpublished results).

Mechanisms which cause loss of IAA production in *P. savastanoi* offer explanations for the spontaneous loss of virulence commonly observed in both plant pathogenic bacteria and fungi. Since loss of virulence is not lethal to most plant pathogens, expression of this trait could be associated with secondary metabolism, much like IAA production in *P. savastanoi.*

REGULATION OF INDOLEACETIC ACID SYNTHESIS AND ITS RELATIONSHIP TO VIRULENCE AND FITNESS OF THE PATHOGEN

As noted in Figure 4, tryptophan monooxygenase fulfills the important function of diverting tryptophan into the production of IAA; the enzyme occupies a key position in not only regulating the production of IAA but also the diversion of tryptophan into secondary metabolism. It is a FAD linked enzyme with a monomer weight of 62 kd, and is strongly inhibited by its product, indoleacetamide ($K_i = 7\ \mu M$) (Hutcheson and Kosuge, 1985). Because the inhibition is competitive with respect to the substrate, L-tryptophan, the cellular pool size of tryptophan is important in regulating the flow of tryptophan into the production of IAA. Thus, the production of the virulence factor, IAA, depends upon the availability of the primary metabolite, L-tryptophan, the production of which is regulated by feedback inhibition of anthranilate synthase by tryptophan. Therefore, mechanisms controlling production and utilization of the amino acid affect expression of virulence. In the normal life cycle of the bacterium, the priority for tryptophan utilization is protein synthesis and secondarily IAA production. Control over the conversion of tryptophan to IAA is provided by strong inhibition of tryptophan monooxygenase by indoleacetamide that is competitively reversed by the substrate, L-tryptophan (Hutcheson and Kosuge, 1985; Kosuge et al., 1966).

That tryptophan pool size limits IAA production in wild type cells is evident since addition of an exogenous source of L-tryptophan boosts IAA production at least 10-fold (Smidt and Kosuge, 1978). One mutant, 2015-5, when compared with its parent, 2015, accumulates 3- to 4-fold higher amounts of IAA in culture without tryptophan supplementation but has comparable levels of tryptophan monooxygenase (Smidt and Kosuge, 1978). The intracellular concentration of tryptophan in this mutant was 35-fold higher than that of its parent strain. When grown with an exogenous source of

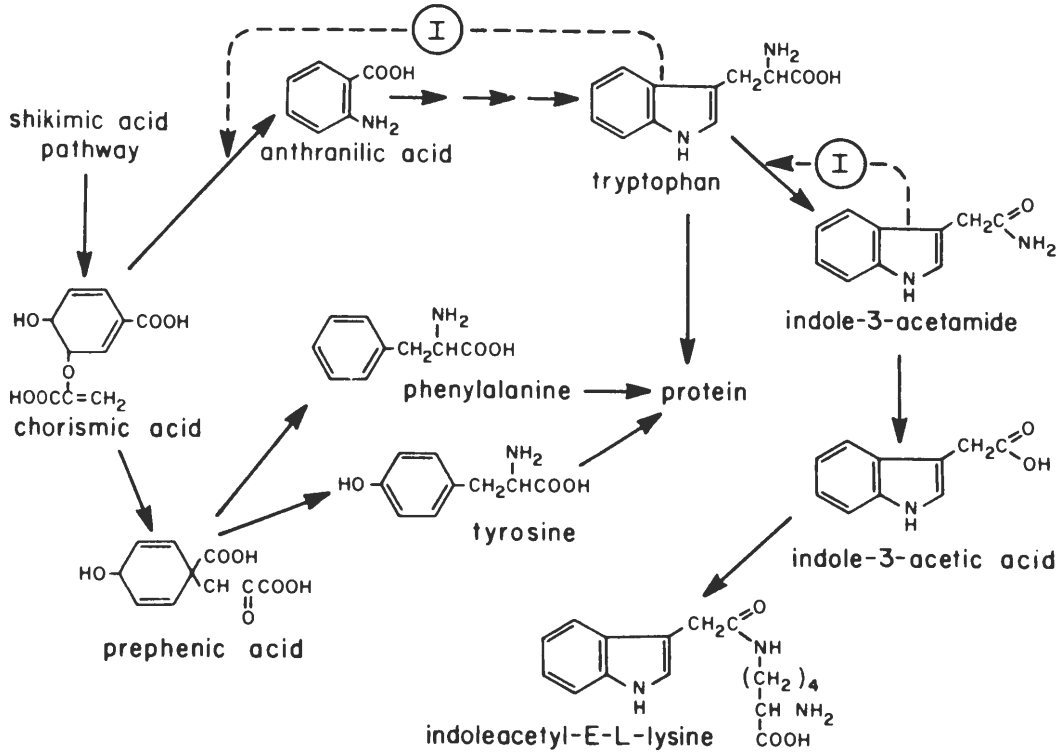


Figure 4. The pathway for indoleacetic acid synthesis in *Pseudomonas savastanoi*. Control of IAA synthesis occurs in part by inhibition of tryptophan monooxygenase by indoleacetamide. Because the inhibition is competitively reversed by the substrate, tryptophan, tryptophan pool size is important in modulating the inhibition by indoleacetamide.

Tryptophan pool size is regulated by feedback inhibition of anthranilate synthase by tryptophan. Other mechanisms, as yet unidentified, may regulate tryptophan and indoleacetic acid synthesis.

L-tryptophan, IAA production was increased but was comparable to the elevated amounts accumulated by parental strains grown in the presence of tryptophan; thus, the super producer phenotype of mutant 2015-5 is expressed only under conditions of limited (controlled) tryptophan supply.

This mutant causes severe symptoms and in some cases causes production of rooty tumors on oleander (Fig. 2). A lowered cytokinin/IAA ratio due to overproduction of IAA by the bacterium appears to be the basis for the rooty tumor phenotype. Although proficient in the production of IAA, the mutant grows somewhat more slowly in oleander tissues compared with its parent wild type (Smidt and Kosuge, 1978); other mutants with very high capacity for IAA production (and potentially highly virulent) fail to grow in host tissue, even though they multiply in culture at wild-type rates and accumulate 10-fold higher concentrations of IAA (L. Glass and T. Kosuge, unpublished results). Apparently, relaxed control of IAA production reduces fitness in this mutant. These mutants should provide insights on the relationship between fitness and altered metabolic regulation.

As noted above, it is known that tryptophan synthesis in *Pseudomonas savastanoi* is regulated, in part, by feedback inhibition of anthranilate synthase by L-tryptophan (Smidt and Kosuge, unpublished results). Mutations to relaxed control of anthranilate synthase will result in increased tryptophan and IAA production and increased virulence. However, the resulting relaxed control of tryptophan synthesis will reduce the efficiency of carbon, nitrogen and energy utilization in the bacterium and may reduce the capacity of the pathogen to compete and to survive under nutrient limitation. Since conditions of nutrient limitation undoubtedly occur comonly in nature, it is expected that increased virulence due to increased IAA production will occur at the expense of decreased fitness of the pathogen. Thus, we propose that metabolic regulation optimizes fitness and virulence in populations of the pathogen.

Clearly, relaxed metabolic regulation cannot explain the relationship between hypovirulence and reduced fitness. In the case of IAA production in *P. savastanoi*, the connection between hypovirulence and fitness perhaps is related to the substrate specificity of tryptophan monooxygenase; the enzyme catalyzes the oxidation of a number of toxic tryptophan analogues including 5-methyltryptophan and halogenated tryptophans. The oxidized products are not toxic to the bacterium. Thus a possible function of tryptophan monooxygenase is the detoxification of toxic tryptophan analogues that may occur in

nature. Indeed, it is known that 7-chlorotryptophan is produced as an intermediate in the production of pyrrolnitrin by certain Pseudomonas species (Leisinger and Margraff, 1979).

MOVABLE ELEMENTS CONTROL EXPRESSION OF VIRULENCE AND SECONDARY METABOLISM

Movable (transposable) elements such as IS-elements in bacteria are commonly associated with changes in phenotype by insertional inactivation of genes (Chandler and Galas, 1985). In *Pseudomonas savastanoi*, this phenomenon is commonly associated with loss of IAA production and virulence in mutants that are selected for resistance to α-methyltryptophan. Loss of virulence in three *iaa*-mutants has been attributed to movable elements IS-51 and IS-52 (Comai and Kosuge, 1983). Although the IS-elements differ significantly in nucleotide sequences (Yamada et al., unpublished results), they nevertheless share the common property of transposition into *iaaM*, causing loss of virulence and IAA production. The site of integration in *iaaM* is marked by the consensus sequence, purine-A-G, in the target gene; however, the integration is not at the same location. Both IS-51 and IS-52 have characteristics in common with those found in other IS-elements; IS-51 has inverted repeats of 26 bp at each end whereas IS-52 has inverted repeats of 12 bp.

Mutations by insertional inactivation are responsible for changes of phenotype in other plant pathogenic bacteria. Loss of tumorigenicity has been observed in *Agrobacterium tumefaciens* by spontaneous insertion of an IS-element in the T-DNA (Garfinkel and Nester, 1980). Garfinkel and Nester (1980) also used Tn5 insertions in the T-DNA to isolate mutants which either were avirulent or caused formation of morphologically altered tumors on tobacco and Kalanchoë plants. Altered tumor morphology has now been attributed to inactivation of T-DNA genes responsible for growth hormone production in tumor tissue (Akiyoshi et al., 1983). Spontaneous loss of virulence in *P. syringae* pv *solanacearum* may be due to insertional inactivation of genes responsible for extracellular polysaccharide production in this bacterium (Staskawicz, 1983).

Transposon mutagenesis is comonly used to isolate avirulent mutants of a number of different species of plant pathogenic bacteria and is a very useful approach for the study of bacterial pathogenesis (Staskawicz, 1983). The mechanism of transposition of IS-elements is under active investigation in a number of laboratories and an

understanding of this process will provide important insights on the molecular basis of variability in plant pathogenic bacteria.

IAA POOL SIZE AND VIRULENCE

Because IAA synthesis is responsible for tumorigenicity and virulence in *Pseudomonas savastanoi*, it follows that systems involved in the conversion of IAA to non-tumorigenic compounds will reduce virulence in the pathogen. Such a role is seen for the system which catalyzes the conversion of IAA to IAA-lysine in oleander strains of *P. savastanoi* (Kosuge, Comai, and Glass, 1983; Hutzinger and Kosuge, 1968). The enzyme catalyzing the synthesis of IAA-lysine from IAA, IAA-lysine synthetase, requires ATP and a divalent metal cation such as Mg^{+2} or Mn^{+2}. The genetic determinant (*iaaL*) for the enzyme is borne on pIAA but is not part of the *iaa* operon. Olive isolates, unlike their counterparts from oleander, are unable to produce IAA-lysine.

IAA-lysine synthetase may help control the pool size of free IAA since oleander strains typically accumulate less IAA in culture than olive strains (L. Glass and T. Kosuge, unpublished results). IAA-lysine is not the end product of IAA metabolism, however; oleander strains convert the compound to its N-acetyl derivative (Evidente et al., 1985) and to other compounds which remain to be identified (Hutzinger and Kosuge, 1968).

RELATIONSHIP TO OTHER TUMORIGENIC SYSTEMS

Production of phytohormones is responsible for crown gall tumorigensis. The role of phytohormone production in crown gall tumor formation was demonstrated in an elegant fashion by Garfinkel and Nester (1980) in their work with Tn5 mutants of *Agrobacterium tumefaciens* that gave rise to leafy and root-forming tumors when inoculated into tobacco plants. The genetic determinants in the T-DNA responsible for phytohormone production have been shown to be gene 1 and 2 (Schröder et al., 1984; Thomashow et al., 1984), which encode enzymes catalyzing IAA synthesis, and gene 4, which encodes an enzyme catalyzing cytokinin production (Akiyoshi et al., 1984; Barry et al., 1984). The pathway for IAA synthesis in crown gall tissue appears to be similar to the one found in *Pseudomonas savastanoi* (Fig. 4) and similarities in gene structure between gene 1 of T-DNA and *iaaM* and gene 2 and *iaaH* are seen in the deduced amino acid sequences (Yamada et al., 1985). Very high homology is evident in the

amino acid sequences involved with the putative FAD binding site in the tryptophan monooxygenase protein.

Such evidence suggests that the genes for IAA synthesis had a common origin in the two tumorigenic systems. Perhaps even more intriguing is the question of the role of the *iaa* operon in bacteria in general. A number of plant pathogenic bacteria not associated with gall formation on their hosts nevertheless have DNA sequences showing strong homology with the *iaaM* and *iaaH*. If these genes are functional in those bacteria, what selection pressure maintains them? As mentioned previously, perhaps their function is to detoxify toxic tryptophan analogues that are produced as antibiotics by competing epiphytic bacteria.

CONCLUSION

Ideas concerning the relationships between metabolic regulation and expression of virulence in plant pathogenic bacteria are mostly based upon intuition rather than experimental evidence; an understanding of the control of secondary metabolism is just beginning. It is clear, however, that this area of research can now be addressed in definitive ways. New approaches that are being developed rapidly in biochemistry and molecular biology seem particularly well-suited for application to the study of plant pathogens. It seems likely that novel mechanisms of metabolic regulation, differing from mechanisms found for the regulation of primary metabolism, will be found for control of secondary metabolism and expression of virulence.

In this paper, we omitted discussions on the regulation of pectate lyase, a virulence factor for soft rot bacteria. The gene (*pat*) which encodes the enzyme is regulated by glucose repression and cAMP much in the same way as the *lac* operon is regulated by *E. coli* (Collmer et al., 1982). Pectate lyase in soft rot bacteria would represent a "catabolic type" virulence gene. The relationships between metabolic regulation, expression of virulence and fitness may be an obvious one which nevertheless requires more proof from future well designed experiments.

LITERATURE CITED

Aharonwitz, Y. 1980. Nitrogen metabolite regulation of antibiotic biosynthesis. Ann. Rev. Microbiol. 34:209-233.

Akiyoshi, D. E. and collaborators. 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc. Natl. Acad. Sci., USA 80:407-411.

——— and collaborators. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci., USA 81:5994-5998.

Atkinson, D. E. 1969. Regulation of enzyme function. Ann. Rev. Microbiol.23:47-68.

Barry, G. F., S. G. Rogers, R. T. Fraley, and L. Brand. 1984. Identification of a cloned cytokinin biosynthetic gene. Proc. Natl. Acad. Sci., USA 81:4776-4780.

Chandler, M. and D. J. Galas. 1985. Studies on the transposition of IA*1*, pp. 53-57. *In*: D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (eds.). Plasmids in bacteria. Plenum Press, New York.

Collmer, A., P. Berman, and M. S. Mount. 1982. Pectate lyase regulation and bacterial soft-rot pathogenesis, pp. 395-422. *In*: M. S. Mount and G. H. Lacy (eds.). Phytopathogenic prokaryotes, vol. I, Academic Press, New York.

Comai, L. and T. Kosuge. 1983a. The genetics of indoleacetic acid production and virulence in *Pseudomonas savastanoi*, pp. 363-366. *In*: A. Puhler (ed.). Molecular genetics of the bacteria-plant interactions, Springer-Verlag, Berlin.

——— and ———. 1983b. Transposable element that causes mutations in a plant pathogenic *Pseudomonas* sp. J. Bacteriol. 154:1162-1167.

——— and ———. 1982. Cloning and characterization of *iaaM*, a virulence determinant of *Pseudomonas savastanoi*. J. Bacteriol. 149:40-46.

——— and ———. 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. J. Bacteriol. 143:950-957.

———, G. Surico, and T. Kosuge. 1982. Relation of plasmid DNA to indoleacetic acid production in different strains of *Pseudomonas syringae* pv *savastanoi*. J. Gen. Microbiol. 128:2157-2163.

Crawford, I. P. and G. V. Stauffer. 1980. Regulation of tryptophan biosynthesis. Ann. Rev. Biochem. 49:163-195.

Dunkle, L. D. 1984. Factors in pathogenesis, pp. 19-41. *In*: T. Kosuge and E. W. Nester (eds.). Plant-microbe interactions-molecular and genetic perspectives, Vol. 1, MacMillan, New York.

Evidente, A., G. Surico, N. S. Iacobellis, and G. Radazzo. 1985. Isolation and structural characterization of N-acetyl-indole-3-acetyle-ε-L-lysine: a new metabolite of indole-3-acetic acid from *Pseudomonas syringae* pv *savastanoi*. Phytochemistry 24:1499-1502.

Garfinkel, D. J. and E. W. Nester. 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. 144:732-743.

Hutcheson, S. W. and T. Kosuge. 1985. Regulation of 3-indoleacetic acid production in *Pseudomonas syringae* pv *savastanoi*. J. Biol. Chem. 260:6281-6287.

Hutzinger, O. and T. Kosuge. 1968. Microbial synthesis and degradation of indole-3-acetic acid. III. The isolation and charcterization of indole-3-acetyl-*E*-L-lysine. Biochemistry 7:601-605.

Kosuge, T., L. Comai, and N. L. Glass. 1983. Virulence determinants in plant-pathogen interactions, pp. 167-177. *In*: R. Goldberg (ed.). Plant molecular biology, Alan R. Liss, Inc., New York.

————, M. G. Heskett, and E. E. Wilson. 1966. Microbial synthesis and degradation of indole-3-acetic acid. I. The conversion of L-tryptophan to indole-3-acetamide by an enzyme system from *Pseudomonas savastanoi*. J. Biol. Chem. 241:3738-3744.

Leisinger, T. and R. Margraff. 1979. Secondary metabolites of the fluorescent Pseudomonads. Microbiol. Rev. 43:422-442.

Schröder, G., S. Waffenschmidt, E. W. Weiler, and J. Schröder. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. 138:387-391.

Smidt, M. and T. Kosuge. 1978. The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. Physiol. Plant Pathol. 13:203-214.

Staskawicz, B. J. 1983. Molecular genetics of plant disease, pp. 199-213. *In*: J. A. Callow (ed.). Biochemical plant pathology, John Wiley, New York.

Thomashow, L. S., S. Reeves, and M. F. Thomashow. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the Agrobacterium Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc. Natl. Acad. Sci. USA 81:5071-5075.

Yamada, T., C. J. Palm, B. Brooks, and T. Kosuge. 1985. Nucleotide sequences of *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc. Natl. Acad. Sci. USA 82:6522-6526.

IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 545-567
Vol. 60, No. 4

PHYSIOLOGICAL ASPECTS OF PLANT-INSECT INTERACTIONS[1]

John C. Reese and Douglas J. Schmidt[2]

ABSTRACT. External excitatory and inhibitory stimuli for host acceptance or rejection are modified by the internal condition of the insect (e.g., hunger). This modificaton can cause a given stimulus to have a greater or lesser amount of "leverage." After acceptance, there is still a "sieve" through which the insect must pass in order to survive, grow, and reproduce. Some of these obstacles include allelochemics that block nutrient availability, poor balance of essential nutrients, defenses of susceptible plants, and less than optimal water content. Neonate larvae may be far more sensitive to some of these obstacles than older larvae.

Index descriptors: plant-insect interactions, allelochemics, neonate sensitivity, insect dietetics, feeding behavior.

INTRODUCTION

While recognizing the difficulty of making generalizations about such a complex field as plant-insect interactions, we will attempt to point out some salient features of the area as we know it today. Examples from our work and from the literature will illustrate some of these points.

Models

For many years, it was thought that the presence of different nutrients in plants might explain the host specificity observed in phytophagous insects. As knowledge expanded, it appeared that differences in nutritional requirements among insect species were not great. Also, plants seemed to vary more dramatically with respect to secondary plant substances or allelochemics than in terms of nutrients. This led Fraenkel (1959) to hypothesize that, given the presence of most essential nutrients in most plants, and given the apparent similarity in nutritional requirements among insects, the

[1]Contribution 85-515-J from the Kansas Agricultural Experiment Station. Research reported here supported by University of Delaware Agricultural Experiment Station Hatch Project 215, Kansas Agricultural Experiment Station Hatch Project 470, and the University of Delaware Research Foundation project Plant Defense Against Insect Attack.

[2]Department of Entomology, Kansas State University, Manhattan, KS 66506.

real explanation of host specificity must lie with the more host-specific allelochemics. This idea served as an extremely important conceptual framework for much research. However, in an attempt to bring post-ingestive processes back into the picture of plant-insect interactions, the chronic effects hypothesis was proposed by Reese and Beck (1976d). They suggested that some allelochemics may have, in addition to immediate effects on survival and feeding behavior, subtle chronic effects on growth rate, utilization of food, and pupation. These post-ingestive effects may serve as a further barrier to host colonization and as "punishment" for behavioral "mistakes." An organism might quickly overcome behavioral deterrents and colonize a new resource if it were not deleterious at the physiological level. The term "dietetics" was used by Beck (1972) to pull together these two concepts and to show that both ideas play a part in host plant suitability. That is, the food ingested must not only contain essential nutrients, but these essential nutrients must be capable of being assimilated and converted into the energy and structural substances required for normal activity and development. Thus, it is recognized that both nutrients and non-nutrients affect host suitability.

Models for the strictly behavioral aspects of host plant acceptance have been discussed for years, but a very intriguing recent one is the "rolling fulcrum" model proposed by Miller and Strickler (1984). It is a mechanical analogue of Dethier's (1982) model for the influence of external and internal factors on insect acceptance or rejection of a candidate host plant. The uniqueness of this model lies in a moveable fulcrum that represents the changes in sensitivity to external excitatory and inhibitory stimuli based on the internal condition of the organism (e.g., hunger vs. satiation).

We have modified the rolling fulcrum model to include post-ingestive phenomena as well as pre-ingestive behavior, and have used it as a conceptual framework to show the relationships between our experiments and certain physiological aspects of plant-insect interactions (Fig. 1). The first component of the model is the rolling fulcrum as described by Miller and Strickler (1984). External stimuli include such things as pH of plant tissues perceived by an insect seeking phloem tissue, as well as factors closer to the surface. Having accepted the plant (we recognize that this is not as clearcut an event as the model would indicate), the insect must be able to ingest and assimilate essential nutrients. The insect must be capable of converting these materials to the energy and structural materials necessary for survival, growth, and reproduction. Allelochemics

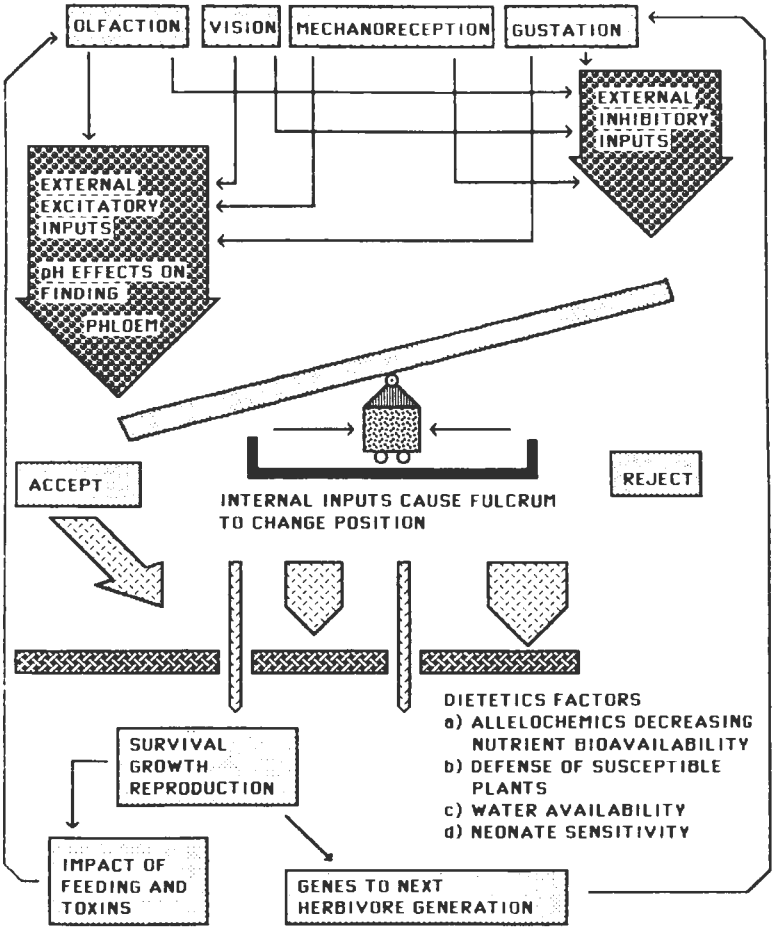


Figure 1. Model for the influence of external and internal factors on insect investment behaviors and the influence of post-ingestive dietetics factors on survival, growth, and reproduction. Modified from Miller and Strickler (1984).

blocking the bioavailability of nutrients, various defenses of even the most susceptible plants, lack of enough moisture, and neonate sensitivity of the insect, form a sieve through which not all individuals pass. Those that survive (thin arrows) these challenges (many others could be added) may pass their genes on to future generations, thus continuing the cycle. Similarly, plants that tolerate insect feeding and the effects of insect-produced toxicants survive to reproduce and ensure the continued availability of the insect's host.

While still incomplete, Figure 1 serves to illustrate the complexity of some physiological aspects of plant-insect interactions.

BEHAVIOR

A broad array of insect behaviors is associated with the utilization of host plants, and the preference for a potential host is mediated through chemical as well as visual and physical clues. Precise levels of sensory stimuli affect the frequency of host finding, examining behavior, and the eventual acceptance or rejection of the plant. Certain combination of these inputs stimulate host finding and feeding, while others are inhibitory and stimulate antagonistic behaviors (Dethier, 1982).

The pre-ingestive components of host plant recognition and utilization have been divided into finding, examining, and consuming (Miller and Strickler, 1984). Many insects perceive host plants at a distance by means of visual and olfactory cues. Flying insects such as the honey bee, *Apis melifera* L., and apple maggot, *Rhagoletis pomenella* (Walsh), are attracted to objects that approximate the size, shape, and hue of their hosts. Likewise, host volitiles attract ovipositing females of the cabbage fly, *Delia brassicae* (Weidemann), and onion fly, *D. antiqua* (Meigen), over substantial distances. At closer range, tactile as well as olfactory cues serve as indicators for host recognition. The cabbage butterfly, *Pieris brassicae* (L.), is a well known example exhibiting typical "drumming" behaviors when in direct contact with its host. Eventually the insect may take test bites or make feeding probes before commencing active consumption to confirm that it has alighted on a proper host (Bell, 1984; Miller and Strickler, 1984; and references therein).

For many insects there are overlapping regions of behavioral stimuli at different distances from the host. Specific stimuli may elicit a range of insect behaviors that observers interpret as a chain of behavioral reactions leading to the eventual acceptance or rejection of the candidate host. Discussion has developed in the literature

concerning the relative importance of one stimulus over another and the significance of receptor selectivity and modality. Sinigrin, as an example, is considered a classical example of a behavioral sign stimulus. The response of many cruciferous feeders suggests that this compound plays an overwhelming role in the recognition of this plant group by some oligophagous insects. This relationship has been used as a model for other insect and plant interactions and promoted the assumption that highly specific receptors for compounds such as sinigrin are the major controlling elements in the suitable recognition of host plants.

Single compounds such as sinigrin, sucrose, and other factors such as pH undeniably exert a strong effect on insect behavior, but gustatory and olfactory receptors are not rigidly specific. The same receptors will generate different activity patterns when exposed to different sets of stimuli, and precise combinations of stimuli are often necessary to elicit desired responses. The peripheral nervous system transduces external stimuli into complex activity patterns that are generated by receptor groups. The chemosensory system, in particular, provides the insect with a specific sensory image of the plant under evaluation. Host plant suitability and insect feeding behavior, then, are mediated by attractive sign stimuli, nutrient availability, and other plant compounds, to include allelochemicals. Preference for one food over another is made on the basis of assessment of these patterns by the central nervous system.

The greenbug, *Schizaphis graminum* (Rondani), is a major pest of cereals and small grains in North America and is the subject of feeding behavior studies in our laboratory. It reproduces parthenogenetically, maturing in six to 18 days. On an acceptable host plant, the adult female will reproduce for 20 to 30 days, larviposting between 50 and 60 nymphs. The winged individuals are not exceptionally strong fliers but are capable of long migrations on windy days (Wadley, 1931). Under favorable conditions, there may be as many as 20 generations of greenbugs per year.

This species is proportionally more injurious than other grain aphid species. Greenbug feeding damage is characterized by a dark red spot at the actual feeding site surrounded by mottled or pale yellow and green tissue. This phenomenon is readily observable in field infestations, where large aggregations (several centimeters in diameter) of greenbugs can be found on the underside of mature sorghum leaves, but has not been characterized chemically. The damage, apparently irreversible, results in localized necrosis wherever the insects have fed. Further, the degree of damage to plant tissue tends

to increase geometrically with increases in the number of greenbugs present (Chatters and Schlehuber, 1951).

Greenbug (biotype E) feeding behavior involves intercellular penetration of plant tissues by the stylets. As the greenbug probes, it deposits a sheath around the stylets composed of solidified salivary secretion. The stylet sheath is normally compressed by the walls of surrounding cells and remains in the plant following withdrawal of the mouthparts. By following the stylet sheaths in properly stained and sectioned plant leaves, one can determine not only the number of probes made by the insects but the depth and success of these efforts. On an artificial liquid diet (Dreyer et al., 1981), where the greenbugs have fed through a Parafilm membrane, the intact sheaths are easily photographed (Fig. 2).

The actual path taken by the stylets to the feeding site is closely controlled and directed by the insect. Searching out the phloem that is hidden beneath several layers of parenchyma and schlerenchyma cells must be a formidable task. As the stylets progress forward, they travel for short distances between the cell wall and the cell membrane and may occasionally enter the protoplasm. Aided by the discharge of salivary enzymes, the stylets probably bring back small amounts of solubilized material to gustatory sensilla in the epipharynx (Backus and McLean, 1982; Miles, 1972). Wensler and Filshie (1969) described apparently chemsensory sensillae anterior and posterior to the precibarial valve of two species of aphids, and McLean and Kinsey (1984) confirmed the presence of gustatory chemoreceptors in the precibarium of the pea aphid, *Acyrthosiphon pisum* (Harris). The greenbug may use a similar method to evaluate the efficiency of its probing efforts.

The orientation of insect stylets to the vascular bundles due to hydrogen ion concentrations was first reported by Fife and Frampton (1936). They measured a pH gradient extending from the epidermis to the phloem of sugar beet (a dicotyledon) and related this to the feeding behavior of the beet leafhopper, *Circulifer tenellus* (Baker). The leafhoppers fed in the phloem tissue, which had a substantially higher pH than the surrounding plant cells. Other discriminating criteria for phloem-feeding insects have been indicated, including carbohydrate concentration (Davidson, 1923) and positive hydrostatic pressure (Mittler, 1957).

On an artificial diet, biotype C and biotype E greenbugs respond to pH in a manner similar to the beet leafhopper (Dreyer et al., 1981). In both choice and no-choice experiments, the biotype E aphids preferred a slightly alkaline diet. This suggests that the pH of their

Figure 2. Greenbug stylet sheaths through Parafilm membrane. Measurement bar is 10 microns.

natural food source (phloem photosynthate) is between 8.0 and 8.5, the optimal pH for laboratory rearing on artificial diet. Direct observations of aphid probing and feeding behavior were made with a binocular microscope from behind the Parafilm membrane. It was also noted that greenbugs on the preferred diet tend to feed for longer periods of time and wandered away from the food less often than did insects on nonpreferred diets.

To determine the pH of the plant tissues, young sorghum plants were cross-sectioned, washed quickly with distilled water, and mounted under a binocular microscope. Microelectrodes for pH measurement (Microelectrodes, Inc., Londonderry, NH 03053) were held in micromanipulator arms and placed into individual plant cells. The pH of the phloem was found to be more alkaline than that of the surrounding cells and, not surprisingly, similar to the pH of the optimal artificial diets.

Once the greenbugs locate the phloem and begin feeding, they apparently become less sensitive to the pH of their food source. On artificial diet, this process occurs within seconds of puncturing the Parafilm membrane and fully inserting the stylets. On a susceptible sorghum plant, it may take the insects as long as 30 minutes to reach the phloem and begin feeding (Campbell et al., 1982). Large groups of greenbugs were placed on diet and allowed to feed undisturbed. At selected intervals, following their introduction to the feeding cages, 40% formic acid was injected into the diet containers. The result of this action was a drop in the pH of the diet from 8.5 to approximately 1.8. In cages where the insects had been given ample time to insert their stylets and begin feeding, few or no behavioral changes were observed. The insects continued to feed and produce honeydew for several hours until they fell to the floor of the cage and died. This suggests that there is a window of sensitivity or a behavioral program that allows them to accurately discriminate dietary pH only while they are probing.

NUTRITIONAL REQUIREMENTS

For a species of insect to survive, its food must, as a minimum, contain the nutrients essential for that species (see Fig. 1; the bioavailability of nutrients will be discussed in a later section.) Nutrition used in this narrow sense (as opposed to the broader term insect dietetics) delineates the minimal nutritional requirements for successful growth, development, and reproduction (Hagen et al., 1984). Identity as a nutrient is not an inherent quality of a particular

compound, but depends upon the physiological capabilities of the ingesting organism. Therefore, a compound that is a nutrient for a fast-growing, immature organism may not be one for a mature individual; a compound that is required at one concentration may be toxic at a tenfold greater concentration (Reese, 1979). Extensive reviews of nutritional requirements have been edited or written by Rodriguez (1972), House (1974), Dadd (1970a, 1970b, 1973, 1977), and Hagen et al. (1984).

Mineral Requirements.

Less work has been done on the specific mineral requirements of insects than for organic nutrients. All insects studied have been shown to require various inorganic chemicals, particularly potassium, magnesium, and phosphate. Although not specifically reported, since many basic biological functions require sodium, calcium, and chloride ions, it would be safe to assume that insects also require them. Insects probably also require iron, zinc, manganese, and copper in low concentrations (Hagen et al., 1984).

Vitamins

The water-soluble B vitamins required by most insects include thiamin, riboflavin, pyridoxine, choline, nicotinamide, folic acid, biotin, and cyanocobalamin (Hagen et al., 1984). Species dependent upon symbiotes may not require exogenous sources of these compounds. Meso-inositol is necessary for proper growth in some species (Dadd, 1961).

Ascorbic acid appears to be essential for most insects that feed on plant tissues, including sap-sucking Homoptera, and leaf-feeding Orthoptera, Coleoptera, and Lepidoptera. However, ascorbic acid has not been shown to be necessary for any Diptera (Hagen et al., 1984). A detailed discussion of the ecological significance of the observed pattern of ascorbic acid requirements, as well as the putative biosynthetic pathways for species that do not require ascorbic acid, is presented by Hagen et al. (1984).

Fat-soluble vitamins such as retinol and β-carotene are needed for visual pigment formation in some species and for growth in others (Brammer and White, 1969; Carlson et al., 1967). α-Tocopherol appears to serve as a generalized antioxidant factor in artificial diets and to be important for reproduction and growth in a few species (Hagen et al., 1984).

Nucleic Acids and Nucleotides

Only Diptera appear to require an exogenous source of nucleic acids or their components, nucleotides, nucleosides, and bases (Hagen et al., 1984).

Sterols

The most stiking differences between the nutritional requirements of vertebrates and insects are their need for dietary sterols. While needed by both groups for cellular membranes, transport lipoproteins, and hormones, only in the case of insects do all species appear to require an exogenous source of sterols. Cholesterol is often used in artificial diets of phytophagous insects, even though it is not found in plant tissue. The reader is referred to Hagen et al. (1984) for a discussion of the sparing (one compound changing the apparent essentiality of another, closely-related compound) effects of various phytosterols.

Essential Fatty Acids

Several polyunsaturated fatty acids are essential as growth factors for most insects that have been studied. For Orthoptera, Lepidoptera, Coleoptera, and Hymenoptera, the fatty acid requirement can be satisfied by linoleic acid or linolenic acid.

Carbohydrates

Although carbohydrates are certainly the major energy source for most insects, fats and proteins also may be involved in energy-producing reactions. Most larvae of phytophagous insects require various sugars or polysaccharides.

Amino Acids

Insects, other than those with symbiotes, require arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Interestingly, these are the same ten amino acids that are essential for the rat and probably many vertebrates. Additional amino acids are required by certain Diptera and Lepidoptera. Some amino acids were thought to be essential before they were shown to be involved in a sparing effect for the essential ones (Hagen et al., 1984). In addition to specific amino acids, total nitrogen appears to be a limiting factor (Slansky and Feeny, 1977).

Water

Although often omitted from lists of essential nutrients, water is the universal biological solvent in which the biochemical reactions of

each cell occur. Most living organisms contain far more water than any other compound or group of compounds. Although most insects are 70-80% water, their food may vary from 1 to over 90% water. Stored-product insects have remarkable abilities to conserve water, whereas phytophagous insects may suffer deleterious effects from low dietary moisture.

The effects of water on digestive efficiency are particularly interesting. Soo Hoo and Fraenkel (1966) suggested that water content of the diet was important to efficiency of conversion in *Prodenia eridania* (Cramer). Hoekstra and Beenakkers (1976) thought that differences in moisture content of their respective host plants accounted for differences in efficiencies of conversion for *Locusta migratoria migratoriodes* (R. & F.). Similarly, Scriber (1977) found that lower moisture levels decreased the efficiency of food conversion in *Hyalophora cecropia* (L.) larvae. Reese and Beck (1978) found that dilution of nutrients by water was not compensated for through increased ingestion in BCW (black cutworm, *Agrotis ipsilon* (Hufnagel) larvae. In these experiments, the efficiency of conversion of assimilated food and the efficiency of conversion of ingested food were negatively correlated with percent dry matter of the artificial diet. However, the optimal percent dry matter of the diet was not the same for both efficiency and growth. Growth clearly depends both upon the amount of dry weight diet ingested and upon efficiency of conversion of ingested food. In the BCW experiments, growth decreased somewhat on the low percent dry matter diets (high efficiency but less dry matter ingested due to dilution), and decreased a great deal as percent dry matter increased above the control diet level (low efficiency and less dry material ingested). Therefore, optimal growth occurred at a percent dry matter very close to that of the control diet level, but below that for maximal dry weight eaten, due to the relationship between dry weight eaten and the efficiency of conversion of ingested food (Reese and Beck, 1978).

ALLELOCHEMICS DECREASING NUTRIENT BIOAVAILABILITY

A second key component of insect dietetics is the effect of defensive allelochemics on insect growth and development. Many amino acid analogs, phenylpropane derivatives, gossypol-like compounds, alkaloids, tannins, lignans, flavonoids, benzoxazolinones, phenolics, benzoic acid derivatives, and benzyl alcohol derivatives have been isolated from plants and have been shown to have deleterious effects on insect growth, development, and reproduction

(Reese and Holyoke, 1986). Although allelochemics are by definition nonnutrients, they have been shown to interact with nutrients. Many of the deleterious metabolic or chronic effects of plant allelochemics may be due to these interactions. Many of the types of interactions discussed in vertebrate nutrition literature may also be important in insect dietetics (Reese, 1979). Certain allelochemics structurally resemble essential nutrients closely enough to compete metabolically. For example, the toxicity of L-canavanine appears to be due to its structural similarity to L-arginine, and thus the formation of defective canavanyl proteins (Rosenthal et al., 1976; Vanderzant and Chremos, 1971; Isogai et al., 1973a, 1973b; Dahlman and Rosenthal, 1975; Rosenthal and Bell, 1979). Allelochemics may block the bioavailability of nutrients by reducing assimilation, efficiency of conversion of assimilated food, or the efficiency of conversion of ingested food (Reese and Beck, 1976a, 1976b, 1976c; Dahlman, 1977; Muthukrishnan et al., 1979; and Erickson and Feeny, 1974).

DEFENSE OF "SUSCEPTIBLE" PLANTS

We have started testing the hypothesis that even susceptible host plants are defended against insect attack when compared to an artificial diet containing low concentrations of defensive allelochemics and having no morphological means of defense. A few species of insects have been observed to have greater fecundity and growth on artificial diets than on preferred plants (Beck, 1974; Whitworth and Poston, 1979a, 1979b; Bailey, 1976; Dittrick and Chiang, 1981).

Glanded cotton varieties are generally slightly more resistant to the bollworm, *Heliothis zea* (Boddie), than their glandless counterparts. Our preliminary experiments utilized *H. zea* on a glanded cotton variety, a glandless variety (presumably more susceptible to bollworm larvae), and on artificial diet. The larvae reared on the glandless variety weighed only 25.1% of the control larvae reared on artificial diet, suggesting that even the supposedly susceptible plant was chemically and/or morphologically quite capable of reducing insect growth.

For later experiments we employed BCW larvae on corn seedlings. While there appear to be species of plants that are even more susceptible to BCW larvae than corn, the susceptibility of corn was not statistically different from that of the least resistant species tested by Busching and Turpin (1977). Further, Wilson et al. (1983) screened 3141 maize entries and found that they all were susceptible to leaf feeding by 3rd-instar larvae. In addition, a total of 3038 entries

were evaluated for leaf feeding by 1st-instar larvae. Only two genotypes showed intermediate levels of resistance, whereas the rest were termed susceptible. Tseng et al. (1984) screened 109 hybrids and 100 inbred lines and found all of them to be susceptible under both greenhouse and field conditions. Out of 2370 maize introductions, Jarvis et al. (1981) found 20 that were intermediate in resistance in terms of leaf feeding, but they were all susceptible in terms of stem cutting.

In our experiments, larvae grown on Pioneer 3368A corn seedlings weighed only 14-15% as much at eight days as larvae fed artificial diet. Funk's G4507A seedlings also inhibited growth compared to artificial diet. By the sixth day of one experiment, weight of the larvae on plants was 8.1% of those on diet. We are currently conducting experiments with several of the apparently still more susceptible species discussed by Busching and Turpin (1977), such as bluegrass (*Poa pratensis* L.), curled dock (*Rumex crispus* L.), lambsquarters (*Chenopodium album* L.), yellow rocket (*Barbarea vulgaris* R. Br.), and rough pigweed (*Amaranthus retroflexus* L.).

SOURCES OF ERROR IN NUTRITIONAL INDICES

In many studies of insect dietetics, nutritional indices have been used to estimate the insect's nutritional efficiency and energy budget. Unfortunately, experimental errors are often made in collecting the data. Small gravimetric and round-off errors can be magnified algebraically by the equations used to calculate the nutritional indices. Seemingly insignificant errors in calculating the initial dry matter of the food, amount of food eaten, and weight of feces produced can substantially alter the assimilation (AD), efficiency of conversion of assimilated food (ECD), and efficiency of all ingested food (ECI). An electronic spreadsheet computer program can be used to simulate the effect of purposefully introduced errors in a nutritional index study.

Schmidt and Reese (1986) derived a hypothetical set of nutritional index data for the BCW on artificial diet. Using the electronic spreadsheet to alter the experimental inputs, they systematically identified a number of error parameters. They concluded that controlling the percentage of food eaten (% FE) was a major factor in obtaining consistant results. Coupled with an accurate estimation of the initial dry matter of the diet, this reduced spurious recordings to negligible levels. If the insects ate a large portion of the food given to them, better results were obtained than when they consumed only a

small fraction of the diet available (Fig. 3). At approximately 80% food eaten (FE), there was less potential for errors in the calculation of percent dry matter of the food than at 20% FE. As the percentage of food eaten decreases, the ECD, AD, and ECI become more prone to error.

Less dramatic, but significant, errors in the nutritional indices may be attributed to collection and measurement of the feces produced. If the fecal pellets are not completely separated from the uneaten food, as is often the case with artificial diets, the AD and ECI will be depressed by approximately 0.5% for each 1.0% error in the dry weight of feces. On intact food sources, such as plant leaves, it may be necessary to separate the feces from cut but uneaten plant matter.

Since insect fecal pellets contain both undigested food and nitrogenous waste products, Bhattacharya and Waldbauer (1972) subtracted the urine content of the feces from the total weight of the fecal pellet. This provided better estimates of assimilation and conversion of assimilated food. Schmidt and Reese (1986) noted that BCW larvae will feed upon their fecal pellets if no other food is available. Growth on fecal pellets is nearly as rapid as growth on diet, suggesting that much of the nutrient content of the diet is not assimilated. The result of fecal feeding on nutritional parameters is an overestimation of the AD and ECI and an underestimation of the ECD.

NEONATE SENSITIVITY

Neonate Sensitivity to Deleterious Effects on Plants

In our effort to develop sensitive bioassays for investigating the effects of plants on insect growth and development, we placed BCW larvae on artificial diet and corn seedlings. Neonate larvae on corn seedlings weighted 1.7 mg seven days later; neonate larvae placed on artificial diet for two days, then fed corn seedlings for five days, weighed 10.2 mg. Thus, even though the larvae started on diet actually spent most (71%) of the experimental period on plants, they weighed six times as much as those kept on plants for the entire experiment. To see if this really were demonstrating neonate sensitivity, a 48-hr "boost" on artificial diet was inserted at various points during the experiment. The data from this experiment (Fig. 4) clearly show that a boost during the first 48 hrs is far more effective at relieving the deleterious effects of the plant than one later in the experiment.

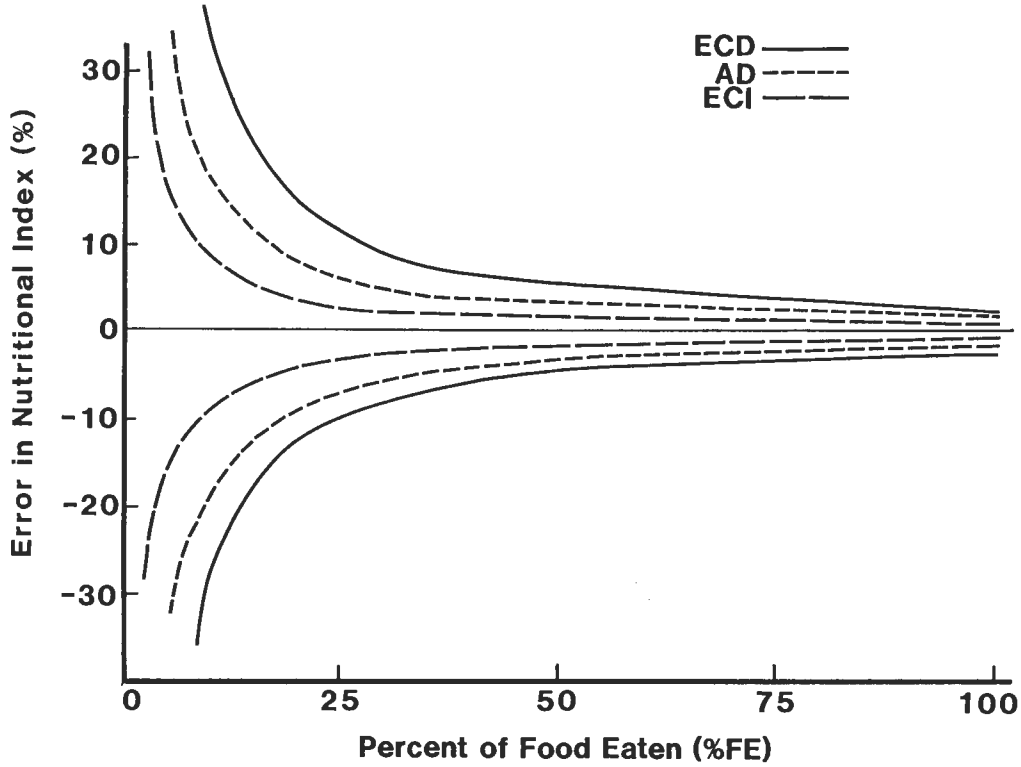


Figure 3. Percent error in AD, ECD, and ECI as a function of percent food eaten (error in calculating the dry matter of the diet = ±0.2%). (AD = assimilation; ECD = efficiency of conversion of assimilated food; ECI = efficiency of conversion of food eaten; FE = food eaten).

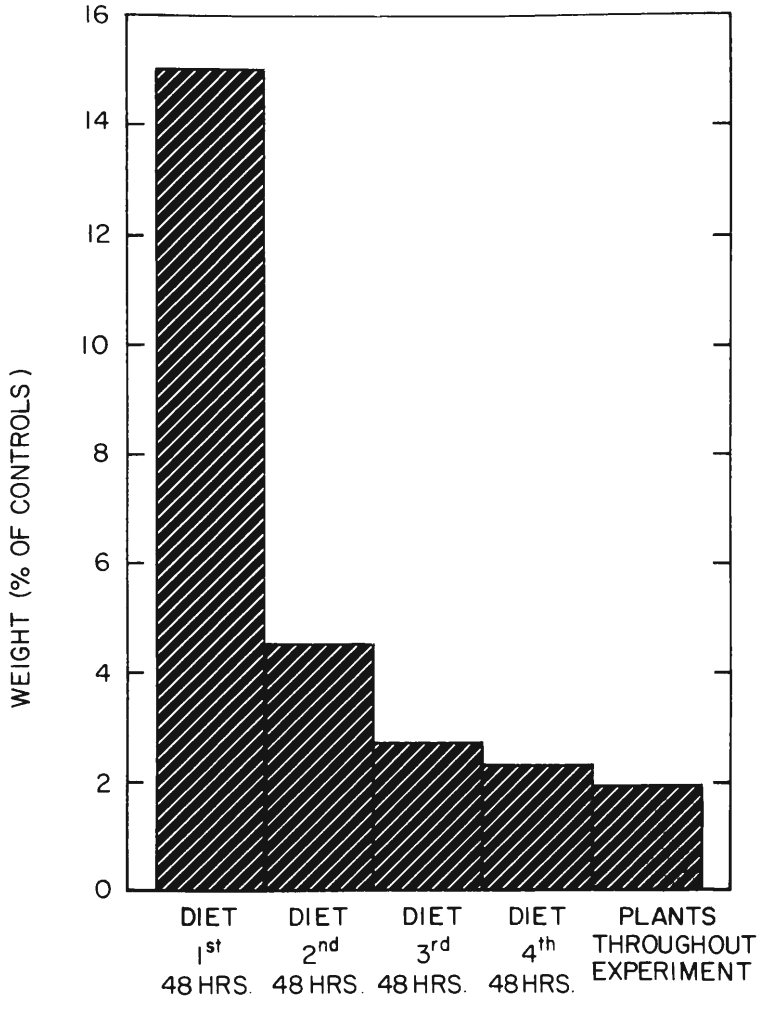


Figure 4. Mean weights of black cutworm larvae fed Funk's G4507A corn seedlings and given a 48 hr "boost" on artificial diet at various times during the experiment. Control larvae were fed artificial diet for the entire experiment. Each bar represents the mean of 17 larvae. All values shown were different from the controls at the $P < 0.001$ level. Actual weights and standard deviations were as follows: 1st 48 hrs, 14.6 $\pm$ 6.2 mg; 2nd 48 hrs, 4.4 $\pm$ 3.0 mg; 3rd 48 hrs, 2.6 $\pm$ 1.7 mg; 4th 48 hrs, 2.4 $\pm$ 1.9 mg; plants throughout, 1.8 $\pm$ 1.4 mg.

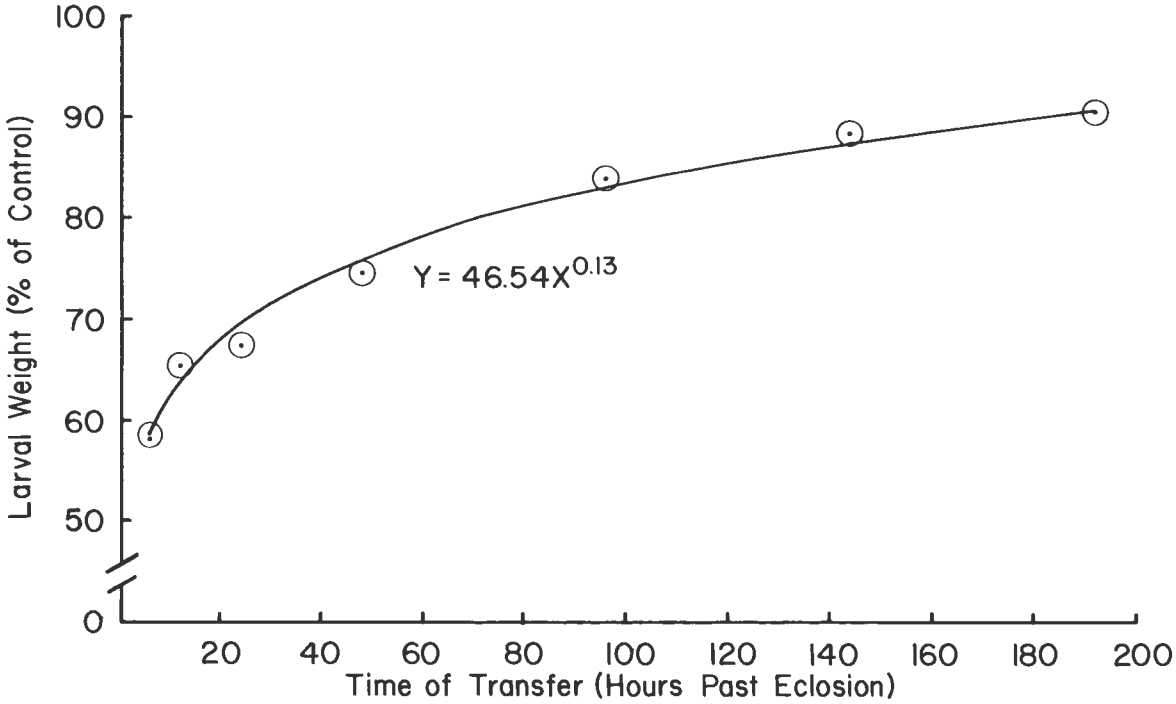


Figure 5. Weight at ten days of black cutworm larvae transferred from one plug of diet to another at various times after eclosion. Each data point represents mean of 20 larvae. The value for 6 hrs past eclosion was significantly different from the controls at $P < 0.001$ level. The values for 12 hrs and 24 hrs were significantly different from the controls at the $P < 0.005$ level. The value for 48 hrs was significantly different from the controls at the $P < 0.01$ level. Actual weights and standard deviations were as follows: 6 hrs, 110.7 ± 54.4 mg; 12 hrs, 123.2 ± 63.1 mg; 24 hrs, 127.1 ± 51.0 mg; 48 hrs, 140.8 ± 53.4 mg; 96 hrs, 158.8 ± 61.9 mg; 144 hrs, 167.1 ± 49.6 mg; 192 hrs, 188.6 ± 65.2 mg.

Table 1. Nutritional performance of disturbed and undisturbed Larvae (** significant at $P < .01$ level; DWE = dry weight eaten; DWG = dry weight gain; AD = assimilation; ECD = efficiency of conversion of assimilated food; ECI = efficiency of conversion of ingested food).

	DWE mg	DWG mg	AD %	ECD %	ECI %
Disturbed Larvae	250.75 ± 82.1	57.92 ± 28.0	35.19 ± 5.4	62.40 ± 5.0	22.11 ± 4.7
Undisturbed Larvae (Control)	376.37 ± 71.6 **	100.74 ± 22.9 **	41.87 ± 2.5 **	63.48 ± 4.3	26.56 ± 2.2 **

Neonate Sensitivity to Handling

Neonate BCW larvae are not only sensitive to the growth-inhibiting effects of plants but also to handling. The physical handling of insect larvae is a common practice in mass-rearing programs. Eggs are usually collected on filter paper or allowed to mature in the adult oviposition chambers. The emerging neonates then are transferred to smaller containers with a paint brush or vacuum aspirator. On artificial diet, immature insects may be transferred two or three times before they emerge as adults.

Weight gain and nutritional index experiments have shown that BCW larvae are quite sensitive to physical disturbance. Neonates that were picked up with a paint brush shortly after eclosion grew more slowly and weighed less than undisturbed larvae. This sensitivity decreases with age but still can be demonstrated many hours following emergence (Fig. 5). In another experiment, a large number of BCW eggs were placed on artificial diet in individual containers. At two days posteclosion, they were picked up individually with a moist paint brush, handled briefly, and replaced on the diet. The weight gain for this group was significantly lower than that for the control group.

Table 1 illustrates that a number of factors were responsible for the lower dry weight gain (DWG) of disturbed larvae at eight days. Physical handling not only reduced ingestion (DWE) but altered the insects' ability to assimilate (AD) and convert food (ECI) to body matter. Conversion of assimilated food (ECD) was the only nutritional parameter unaffected by handling; i.e., a larger percentage of digested food was not metabolized for energy. Reduced weight gain, then, cannot be attributed solely to an interruption of feeding. Handling may diminish the insect's oxidative metabolism or cause internal damage to the digestive apparatus and should be avoided when working with neonate larvae.

LITERATURE CITED

Backus, E. A. and D. L. McLean. 1982. The sensory systems and feeding behavior of leafhoppers. 1. The aster leafhopper, *Macrosteles fascifrons* Stäl (Homoptera, Cicadellidae). J. Morphol. 172:361-379.

Bailey, C. G. 1976. A quantitative study of consumption and utilization of diets in the bertha armyworm, *Memestra configurata* (Lepidoptera: Noctuidae). Canad. Entomol. 108:1316-1326.

Beck, S. D. 1972. Nutrition, adaptation and environment, pp. 1-6. *In*: J. G. Rodriguez (ed.). Insect and mite nutrition. North-Holland Publ. Co., Amsterdam.

———. 1974. Theoretical aspects of host plant specificity in insects, pp. 290-311. *In*: F. G. Maxwell and R. A. Harris (eds.). Proceedings of the Summer Institute of Biological Control of Plant Insects and Diseases. Univ. Press of Mississippi, Jackson, MS.

Bell, W. J. 1984. Chemo-orientation in walking insects, pp. 93-109. *In*: W. J. Bell and R. T. Cardé (eds.). Chemical ecology of insects. Sinauer Assoc. Sunderland, MA.

Bhattacharya, A. K. and G. P Waldbauer. 1972. The effect of diet on the nitrogenous end products excreted by larval *Tribolium confusum* with notes on correction of A.D. and E.C.D. for fecal urine. Entomol. Exp. Appl. 15:238-247.

Brammer, J. D. and R. H. White. 1969. Vitamin A deficiency: Effect on mosquito eye ultrastructure. Science 163:821-823.

Busching, M. K. and F. T. Turpin. 1977. Survival and development of black cutworm (*Agrotis ipsilon*) larvae on various species of crop plants and weeds. Environ. Entomol. 6:63-65.

Campbell, B. C. and collaborators. 1982. Probing behavior of the greenbug (*Schizaphis graminum*, biotype C) on resistant and susceptible varieties of sorghum. Entomol. Exp. Appl. 31:140-146.

Carlson, S. D., H. R. Steeves, III, J. S. VandeBerg, and W. E. Robbins. 1967. Vitamin A deficiency: Effect on retinal structure of the moth, *Manduca sexta*. Science 158:268-270.

Chatters, R. M. and A. M. Schlehuber. 1951. Mechanisms of feeding of the greenbug (*Toxoptera graminum* Rond.) on *Hordeum*, *Avena*, and *Triticum*. Okla. Agr. Expt. Tech. Bull. No. T-40. 18 pp.

Dadd, R. H. 1961. The nutritional requirements of locusts-IV. Requirements for vitamins of the B complex. J. Insect Physiol. 6:1-12.

———. 1970a. Arthropod nutrition. Chem. Zool. 5:35-95.

———. 1970b. Digestion in insects. Chem. Zool. 5:117-145.

———. 1973. Insect nutrition: Current developments and metabolic implications. Ann. Rev. Entomol. 18:381-420.

———. 1977. Qualitative requirements and utilization of nutrients: Insects. CRC Handbook series in nutrition and food. 1:305-346. CRC Press, Cleveland, OH.

Dahlman, D. L. 1977. Effect of L-canavanine on the consumption and utilization of artificial diet by the tobacco hornworm, *Manduca sexta*. Entomol. Exp. Appl. 22:123-131.

——— and G. A. Rosenthal. 1975. Non-protein amino acid-insect interactions. I. Growth effects and symptomology of L-canavanine consumption by tobacco hornworm, *Manduca sexta* (L.). Comp. Biochem. Physiol. 51A:33-36.

Davidson, J. 1923. Biological studies of *Aphis rumicis*. The penetration of plant tissues and the source of the food supply of aphids. Ann. Appl. Biol. 10:279-284.

Dethier, V. G. 1982. Mechanisms of host-plant recognition. Entomol. Exp. Appl. 31:49-56.

Dittrick, L. E. and H. C. Chiang. 1981. Differences in the development response of the European corn borer reared on corn plant vs. meridic diet under greenhouse conditions. Environ. Entomol. 10:889-892.

Dreyer, D. L., J. C. Reese, and K. C. Jones. 1981. Aphid feeding deterrents in sorghum: Bioassay, isolation, and characterization. J. Chem. Ecol. 7:273-284.

Erickson, J. M. and P. Feeny. 1974. Sinigrin: A chemical barrier to the black swallowtail butterfly, *Papilio polyxenes*. Ecol. 55:103-111.

Fife, J. M. and V. L. Frampton. 1936. The pH gradient extending from the phloem into the parenchyma of the sugarbeet and its relation to the feeding behavior of *Eutettix tenellus*. J. Agr. Res. 53:581-593.

Fraenkel, G. S. 1959. The raison d'être of secondary plant substances. Science 129:1466-1470.

Hagen, K. S., R. H. Dadd, and J. C. Reese. 1984. The food of insects, pp. 79-112. *In*: C. B. Huffaker and R. L. Rabb (eds.). Ecological entomology. John Wiley, New York.

Hoekstra, A. and A. M. T. Beenakkers. 1976. Consumption, digestion, and utilization of various grasses by fifth-instar larvae and adults of the migratory locust. Entomol. Exp. Appl. 19:130-138.

House, H. L. 1974. Nutrition, pp. 1-62. *In*: M. Rockstein (ed.) The physiology of insecta (2nd edition). Acad. Press, New York.

Isogai, A., S. Murakoshi, A. Suzuki, and W. Tamura. 1973a. Growth and inhibitory effects of phenylpropanoids in nutmeg on silkworm larvae. J. Agr. Chem. Soc. Japan 47:275-279.

————, ————, ————, and ————. 1973b. Isolation from "Astragali Radis" [sic] of L-canavanine as an inhibitory substance to metamorphosis of silkworm, *Bombyx mori* L. J. Agr. Chem. Soc. Japan 47:449-453.

Jarvis, J. L., W. D. Guthrie, and J. C. Robbins. 1981. Evaluation of maize introductions for resistance to black cutworm larvae. Maydica 26:219-225.

McLean, D. L. and M. G. Kinsey. 1984. The precibarial valve and its role in the feeding behavior of the pea aphid, *Acyrthosiphon pisum*. Bull. Entomol. Soc. Am. 30:26-31.

Miles, P. W. 1972. The saliva of Hemiptera. Adv. Insect Physiol. 9:183-256.

Miller, J. R. and K. L. Strickler. 1984. Finding and accepting host plants, pp. 127-157. *In*: W. J. Bell and R. T. Cardé (eds.). Chemical ecology of insects. Sinauer Assoc. Sunderland, MA.

Mittler, T. E. 1957. Studies of the feeding and nutrition of *Tuberlachnus salignus*. 1. The uptake of phloem sap. J. Exp. Biol. 3:334-341.

Muthukrishnan, J., S. Matharan, and K. Venkatasubbu. 1979. Effect of caffeine and theophylline on food utilization and emergence in *Danaus chrysippus* (Lepidoptera: Danaidae). Entomon 4:309-312.

Reese, J. C. 1979. Interactions of allelochemicals with nutrients in herbivore food, pp. 309-330. *In*: G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their interaction with secondary plant metabolites. Acad. Press, New York.

——— and S. D. Beck. 1976a. Effects of plant allelochemics on the black cutworm, *Agrotis ipsilon*; Effects of p-benzoquinone, hydroquinone, and duroquinone on larval growth, development, and utilization of food. Ann. Entomol. Soc. Am. 69:59-67.

——— and ———. 1976b. Effects of plant allelochemics on the black cutworm, *Agrotis ipsilon*; Effects of catechol, L-dopa, dopamine, and chlorogenic acid on larval growth, development, and utilization of food. Ann. Entomol. Soc. Am. 69:59-67.

——— and ———. 1976c. Effects of plant allelochemics on the black cutworm, *Agrotis ipsilon*; Effects of resorcinol, phloroglucinol, and gallic acid on larval growth, development, and utilization of food. Ann. Entomol. Soc. Am. 69:999-1003.

——— and ———. 1976d. Effects of certain allelochemics on the growth and development of the black cutworm. Symp. Biol. Hung. 16:217-221.

——— and ———. 1978. Interrelationships of nutritional indices and dietary moisture in the black cutworm (*Agrotis ipsilon*) digestive efficiency. J. Insect Physiol. 24:473-479.

——— and C. W. Holyoke, Jr. 1986. Allelochemics affecting insect growth and development. Handbook series on naturally occurring pesticides. Vol. 2, Insect growth regulators. CRC Press, Cleveland. (In Press).

Rodriquez, J. G. (ed.). 1972. Insect and mite nutrition. North-Holland Publ. Co., Amsterdam. 702 p.

Rosenthal, G. A. and E. A. Bell. 1979. Naturally occurring, toxic nonprotein amino acids, pp. 353-385. *In*: G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their interaction with secondary plant metabolites. Acad. Press, New York.

———, D. L. Dahlman, and D. H. Janzen. 1976. A novel means of dealing with L-canavanine, a toxic metabolite. Science 192:256-258.

Schmidt, D. J. and J. C. Reese. 1986. Sources of error in nutritional index studies of insects on artificial diets. J. Insect Physiol. 32:193-198.

Scriber, J. M. 1977. Limiting effects of low leaf-water content on nitrogen utilization, energy budget and larval growth of *Hyalophora cecropia* (Lepidoptera: Saturnidae). Oecologia (Berl.) 28:269-287.

Slansky, F., Jr. and P. Feeny. 1977. Stabilization of the rate of nitrogen accumulation by larvae of the cabbage butterfly on wild and cultivated food plants. Ecol. Monogr. 47:209-228.

Soo Hoo, C. F. and G. Fraenkel. 1966. The consumption, digestion, and utilization of food plants by a polyphagous insect, *Prodenia eridania* (Cramer). J. Insect Physiol. 12:711-730.

Tseng, C. T., J. J. Tollefson, and W. D. Guthrie. 1984. Evaluation of maize single-cross hybrids and inbred lines for resistance to 3rd-instar black cutworm larvae (Lepidoptera: Noctuidae). J. Econ. Entomol. 77:565-568.

Vanderzant, E. S. and J. H. Chremos. 1971. Dietary requirement of the bollweevil for arginine and the effect of arginine analogues on growth and on the composition of the body amino acids. Ann. Entomol. Soc. Am. 64:480-485.

Wadley, F. M. 1931. Ecology of *Toxoptera graminum*, especially as to factors affecting importance in the northern United States. Ann. Entomol. Soc. Am. 24:325-395.

Wensler, R. J. and B. K. Filshie. 1969. Gustatory sense organs in the food canal of aphids. J. Morph. 129:473-492.

Whitworth, R. J. and F. L. Poston. 1979a. A thermal unit accumulation system for the southwestern corn borer. Ann. Entomol. Am. 72:253-255.

———— and ————. 1979b. Growth and development of the southwestern corn borer (Lepidoptera: Pyralidae) on corn. Proc. N. Centr. Br. Entomol. Soc. Am. 33:22

Wilson. R. L., J. L. Jarvis, and W. D. Guthrie. 1983. Evaluation of maize for resistance to black cutworm larvae. Maydica 28:449-453.

IOWA STATE JOURNAL OF RESEARCH / MAY, 1986 569-594
Vol. 60, No. 4

GENETIC STUDIES OF FERTILITY AND PATHOGENICITY IN *MAGNAPORTHE GRISEA (PYRICULARIA ORYZAE)*

Barbara Valent, Mark S. Crawford, Carolyn G. Weaver and Forrest G. Chumley[1]

ABSTRACT. Crosses between field isolates of *Magnaporthe grisea* (anamorph, *Pyricularia oryzae* Cav. and *Pyricularia grisea*) have led to the development of fertile laboratory strains that infect goosegrass and/or weeping lovegrass. These strains can now be utilized in a rigorous genetic analysis of host species specificity and general pathogenicity. Attempts to improve the fertility of rice pathogens for the goal of undertaking a genetic analysis of host cultivar specificity have so far been unsuccessful. Crosses between *M. grisea* strains that infect rice, goosegrass or weeping lovegrass demonstrate that host species specificity differences between field isolates of this fungus may either have a complex genetic basis or a simple genetic basis. Crosses between a field isolate of the pathogen that infects weeping lovegrass and a field isolate that infects goosegrass have made it possible to identify a single gene difference that determines pathogenicity toward weeping lovegrass and a second unlinked single gene difference that determines pathogenicity toward goosegrass.

Index descriptors: *Pyricularia oryzae*, *Magnaporthe grisea*, rice blast, genetic analysis, host-pathogen interactions.

INTRODUCTION

The *Pyricularia* (teleomorph, *Magnaporthe grisea*, Barr, 1977; Yaegashi and Udagawa, 1978a; Yaegashi and Udagawa, 1978b) are fungal plant pathogens with unique promise as a system for the study of host-parasite interactions. The *Pyricularia* are pathogens of many grass species, but individual isolates exhibit a limited host range, infecting one, or at most a few, grass species (Asuyama, 1965; Kato, 1978). Strains that infect grasses other than rice have been designated *P. grisea*, while those that infect rice have been designated *P. oryzae*. However, *P. oryzae* and *P. grisea* are morphologically indistinguishable, and they are interfertile (Yaegashi and Hebert, 1976a; Tanaka et al., 1979; this paper). We now refer to all

[1]Department of Chemistry, Campus Box 215, University of Colorado, Boulder, Colorado 80309. Present address of Valent and Chumley: E. I. Du Pont De Nemours and Co., Inc., Central Research and Development Department, Experimental Station, E402/2208, Wilmington, DE 19898. Present address of Crawford: Institute of Biological Chemistry, Washington State University, Pullman, WA 99163.

strains by the name of the perfect state, *Magnaporthe grisea*. The fungus is a filamentous, heterothallic Ascomycete, and it grows on defined medium, a trait that facilitates both genetic and biochemical analysis. *P. oryzae* (Cav.) is an important pathogen of rice (*Oryza sativa*), causing a disease of leaves, nodes and inflorescence called blast. Hundreds of races have been distinguished among isolates of *P. oryzae* according to the spectrum of rice cultivars they can infect (Latterell et al., 1965; Atkins et al., 1967; Kiyosawa, 1976; Yamada et al., 1976; Ou, 1980). Thus, *M. grisea* offers an excellent system for studying genetic and biochemical determinants of host species specificity, cultivar specificity, and general pathogenicity.

Genetic analysis of electrophoretic enzyme variants (Leung, 1984), nutritional mutants (Waki et al., 1983; Nagakubo et al., 1983b; 1983c), drug resistance mutants (Taga et al., 1978; 1979; 1982;), pigmentation mutants (Nagakubo et al., 1983a) and host specificity variants (Yaegashi, 1978; Yaegashi and Asaga, 1981) of *M. grisea* has been accomplished using field isolates of the fungus. However, such analyses have been limited by the low fertility of field isolates. Problems encountered have included a failure to produce ascospores, or poor germination of ascospores, and rapid loss of fertility as strains were subcultured. Rigorous genetic analysis of *M. grisea* will require the development of fertile laboratory strains that can be crossed reproducibly to yield totally viable ascospores. Ideally, these strains should be derived from a single genetic background and should infect rice, as well as other grasses.

The development of fertile laboratory strains of a plant pathogen is complicated by the need to retain pathogenicity phenotypes. Thus, an understanding of both the inheritance of fertility and the inheritance of pathogenicity is essential for efforts to develop useful laboratory strains of *M. grisea*. We report here investigations of the inheritance of fertility and pathogenicity in three separate sets of crosses between Japanese field isolates; all three involve the hermaphroditic finger millet (*Eleusine coracana*)- and goosegrass (*Eleusine indica*)-infecting field isolate, WGG-FA40, as one of the parents. This strain has been crossed with a second goosegrass pathogen, a weeping lovegrass (*Eragrostis curvula*) pathogen, and a rice pathogen. These crosses have resulted in several fertile laboratory strains that infect goosegrass and/or weeping lovegrass. In our hands, all field isolates that infect rice are female sterile, and all rice-infecting progeny derived in crosses remain female sterile. Results from these crosses confirm that *P. grisea* and *P. oryzae* strains are interfertile: viable

progeny from crosses between the two "species" can be crossed back to either parent. Thus, we believe that there should be no species distinction between strains of the pathogen that infect rice and those that infect grasses other than rice. Finally, the results reported here demonstrate that host species specificity differences between field isolates of *M. grisea* in some cases have a simple genetic basis, and in other cases, have a complex genetic basis.

MATERIALS AND METHODS

Strains

Our research was initiated in July, 1982, when one of us (BV) collected fresh field isolates of *M. grisea* from rice and other grasses with Dr. Hajime Kato in Japan. In addition to these freshly collected isolates, we have received several hundred *M. grisea* strains from scientists in Japan, the Philippines, India, and the United States. In cases where the name of a strain has already appeared in publications, we have retained that name. New field isolates have been named using a number preceded by "O-" for field isolates that infect rice and by "G-" for field isolates that infect other grasses. Strains designated "CP-" are strains that have been produced in the laboratory by means other than genetic crosses.

The *M. grisea* field isolates used in this study were: SM81-4 and WGG-FA40 (finger millet and goosegrass pathogens), K76-79 (a weeping lovegrass pathogen) and Ken60-19 (a rice pathogen), all generously provided by H. Yaegashi; Ina168 (a rice pathogen), generously provided by H. Kato; and O-42 (a rice pathogen), collected by H. Kato and B. Valent in Tochigi Prefecture, Japan. These isolates originated from lesions either on finger millet, weeping lovegrass, or rice in Japan.

Strains designated by a three-part number are single ascospore progeny of genetic crosses. The first part of the strain designation is the cross serial number, the second is the ascus number, and the third is the ascospore number. Strain names in which the ascus number has been replaced with "R" indicate random ascospore cultures. In some crosses, the germination of ascospores is so rare that we do not bother to separate the individual ascospores in an ascus. Any viable ascospores in a single ascus are allowed to germinate and grow together, yielding a culture that may be mixed. All such cultures are eventually purified by isolating single conidia. These single ascus cultures are designated by a two-part number such as 4098-250, which includes the cross serial number and the ascus number.

Formerly the two mating types of *M. grisea* were referred to as "A" and "a" (Kato et al., 1976; Yaegashi and Nishihara, 1976). In order to use a consistent nomenclature at all loci and to avoid implying a dominance relationship between these alleles, we refer to "A" as *mat1-1* and "a" as *mat1-2*.

Establishment of a Permanently Stored Collection of *Magnaporthe grisea* Strains

Previous laboratory studies of *M. grisea* have been plagued by the loss of fertility and pathogenicity because the strains were maintained by serial transfers on slants and Petri dishes. Our permanent storage system for *M. grisea* stains has overcome this problem. Many strains are stored dried and frozen in leaf lesions collected from infected plants. Strains that conidiate abundantly have been stored as conidial suspensions dehydrated on silica gel according to the method of Perkins (1962). Finally, almost all our strains have been stored by drying and freezing filter paper discs (13 mm diameter, S and S #597) on which mycelia have been allowed to grow while the disc was supported on a nutrient agar medium. After the fungus has fully penetrated the filter paper, the paper is peeled from the plate and air dried. The papers then are enclosed in sterile glassine stamp envelopes and filed in water-proof boxes at -20C. Important strains are maintained in at least two separate storage collections. Strains are removed from storage immediately before each experiment.

Media

All media used in this study were solidified with 1.5% agar unless otherwise stated.

Minimal: 1% sucrose, Vogel's N Salts (Vogel, 1964), 1 mg/l thiamine, 5 μg/l biotin.

Complete: 1% sucrose, 0.6% yeast extract, 0.6% casein enzymatic hydrolysate.

Misato-Hara medium: 1% soluble starch, 0.2% yeast extract (Taga et al., 1982).

Oatmeal: 50 g of rolled oats are heated in 500ml of water at 70C for one hour, then filtered through cheesecloth, and the volume of the filtrate is adjusted to one liter with water.

Genetic Crosses

This pathogen is a Pyrenomycete; the events of meiosis transpire within a flask-shaped fruiting body known as a perithecium. The mating

behavior of *M. grisea* suggests that strains of either mating type normally function as self-sterile hermphrodites. Mating compatibility is determined by alleles of the mating type locus, but any single mating event involves a male and a female parent. The capacity for male or female mating proficiency is independent of mating type. Sexual crosses of prototrophic stains are performed by pairing strains of opposite mating type on oatmeal agar (Yaegashi and Hebert, 1976b). Usually, mycelial plugs of the parents are placed about 3-4 cm apart and allowed to grow toward one another at 21C under continuous cool white fluorescent light. Perithecia form at the intersection of growth between the two strains. When two hermaphrodites of opposite mating type are placed on oatmeal plates and allowed to grow together, a distinct double row of perithecia forms (Figure 1). In the most fertile crosses, asci containing viable ascospores appear approximately 13 days after the strains are paired. Crosses involving one parent that infects rice typically take a month to produce asci containing viable ascospores. For tetrad analysis, asci are released from the perithecium using a pair of fine forceps. In good crosses, the ascus walls lose their integrity about 20 minutes after the asci are released from the perthecium. The eight spores of an ascus are separated by hand on 4% water agar using a finely drawn glass needle and a 50X Wild stereomicroscope. In crosses between highly fertile strains, mature perithecia contain loose ascospores. Random ascospore analysis can be easily accomplished by releasing the loose ascospores and separating them on 4% water agar. Often in crosses between strains of low fertility, the ascus walls remain intact. Digestion with the cell wall-degrading enzyme, Zymolyase (Kirin), sometimes aids both tetrad and random ascospore analysis in such crosses. Germinated ascospores are transferred from the 4% water agar to complete medium in wells of Falcon 3047 Tissue Culture Plates. A rich medium inhibits conidiation and therefore reduces cross-contamination. Cellulose filter paper disks (13 mm diameter, S and S #597) are placed in the wells. When the fungus has completely grown into the filter paper, the paper is dried and frozen. This storage technique is simple enough that all progeny from crosses can be stored immediately, thus eliminating chances for degradation of strains due to subculturing.

Testing the Pathogenicity of Strains

Conidia are collected from cultures growing on oatmeal agar plates, autoclaved corn leaves or Misato-Hara medium by washing with a sterile 0.025% solution of Tween 20 detergent. The conidial

Figure 1. Double row of perithecia produced in the mating of two hermaphrodites. This photograph shows the intersection of growth on oatmeal agar of a wild type *Magnaporthe grisea* strain and a strain that carries a pigment mutation. The necks of the perithecia produced are seen protruding from the agar. The black perithecia produced by the wild type strain are clearly distinct from the red perithecia produced by Buf$^-$ mutants. The two types of perithecia are not intermingled, but form two distinct rows. The row of perithecia nearest the point of inoculation of each parent represent mating events in which that parent served as the female partner.

suspension is counted and adjusted to a standard titer. For example, five rice plants growing in a single five-inch plastic pot are routinely infected using a 4 ml suspension containing 5×10^5 conidia per ml. Rice plants are infected at the three- to four-leaf stage. The conidial

suspension to be tested is applied to the plants using an artist's air brush, which produces a fine mist. Infected plants are then sealed inside a plastic bag and left in the dark for 16 hours at 24C. These highly humid conditions are required for the fungus to penetrate the leaves of the host plants. After 24 hours, the bags are removed, and the plants are placed in the greenhouse at 26-28C. Infection types are scored after five to seven days as defined by Latterell et al. (1965). The infection types are scored by examining the youngest leaf expanded at the time of inoculation, as this appears to be the most susceptible plant tissue.

The following criteria distinguish the standard infection types: Type 0, no evidence of infection; Type 1, brown pinpoint lesions; Type 2, small irregularly shaped brown lesions 0.5-1.0 mm in diameter and possessing a small but distinct center; Type 3, some Type 2 lesions interspersed with small eyespot-shaped lesions 2-3 mm in diameter with gray centers surrounded by brown necrotic rings; Type 4, large grayish eyespot-shaped lesions that, with sufficient moisture, enlarge as much as 15 mm in length; Type 5, lesions at first similar to Type 4, but enlarging and coalescing more rapidly. Type 1 brown pinpoint lesions most likely represent the hypersensitive death of tissue in contact with the potential pathogen. Types 0, 1, and 2 are resistant or incompatible interactions, even though the pathogen can sometimes be re-isolated from such lesions. Type 3 is considered an intermediate response. Types 4 and 5 are susceptible interactions.

Infected plants do not produce conidia in the greenhouse if the relative humidity is maintained below 90%. The pathogen can be re-isolated from diseased plants by placing excised tissue on wet filter paper or on 2% water agar plates. Tissue containing Type 4 and Type 5 lesions conidiates heavily within 12 to 24 hours after placing it in such a humid environment. Conidia are harvested by touching the tops of conidiophores with the sharp point of a 4% water agar sliver (as demonstrated by F. Latterell, personal communication) or with a small wire loop containing 0.025% Tween 20 solution.

The standard rice cultivar used in tests of pathogenicity was M9, a cultivar with no known resistance to blast. Seed was generously provided by Dr. M. Morse of the California Cooperative Rice Research Foundation in Biggs, California.

Hosts other than rice were inoculated with fungus in the same manner as described above. Weeping lovegrass seed was obtained from Valley Seed Service in Fresno, California. Plants are infected at two weeks of age, when they have reached the five leaf stage. Goosegrass seed was obtained from Azlin Weed Seed Service in Leland Mississippi.

Goosegrass is infected at the four to five leaf stage. Weeping lovegrass typically shows an all-or-nothing response to infection by different strains of the fungus; i.e., plants are either unblemished by the fungus, or they are completely killed. All weeping lovegrass plants in a pot respond in the same manner. Goosegrass infection is more difficult to score. The several plants in a single pot sometimes show a range of responses to the fungal inoculum. This may reflect heterogeneity in the seed, a possibility that could be investigated by increasing the seed of selected individual plants. Infected goosegrass plants never show the devastating symptoms observed with weeping lovegrass; a range of lesion types similar to those described above for infected rice plants is detected. As controls in each particular pathogenicity test, we include uninoculated plants sprayed with 0.025% Tween 20 solution, as well as plants inoculated with known pathogenic and nonpathogenic *M. grisea* strains.

Judging fertility of *Magnaporthe grisea* strains

The fertility of a strain is a complex characteristic. An assessment of fertility must consider (1) the ability to function as a hermaphrodite, (2) the number of perithecia produced, and (3) the number of viable ascospores contained in each perithecium. First, we record the number of perithecia produced by each parent strain in a cross. In most crosses, the parental strain responsible for producing a perithecium can be distinguished by observing its location on the mating plate. Then we release asci from perithecia and spread them on a 4% water agar plate. Ascospores germinate within 2-12 hours after the asci are released from the perithecium. The number of germ tubes provides an indication of the number of viable ascospores in each ascus.

All aspects of the apparent fertility of a strain can be greatly affected by the choice of a mating partner; in many respects, it is most accurate to describe the fertility of a particular genetic cross, thus referring to both parents simultaneously. Nevertheless, a useful estimation of the average fertility of a strain emerges after it has been mated with a variety of partners.

Production of Pigment Mutants

Buf$^-$ mutants of *M. grisea* were isolated following mutagenesis with UV light. Conidia were diluted to 1×10^6/ml in sterile 0.025% Tween 20. Five ml of this spore suspension was stirred in a 3.5 cm petri dish while being irradiated with UV light for four minutes (Ultraviolet Products Mineralight Model No. UVG-11, placed nine cm

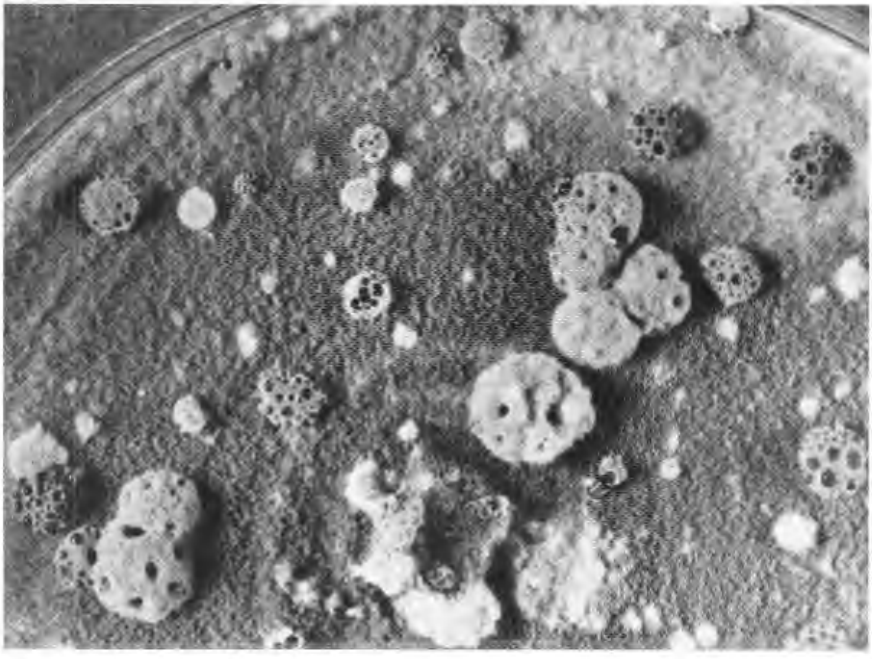


Figure 2. Mosaic morphology of WGG-FA40. This morphology is associated with fresh field isolates of *M. grisea*.

above the conidial suspension). This treatment kills 93-98% of the conidia. The survivors were plated to identify buff-colored mutants.

RESULTS

Instability of *M. grisea* Strains During Continuous Subculture

M. grisea strains have been reported to be unstable with regard to morphology, fertility, and pathogenicity (Taga et al., 1979; Nagakubo et al., 1983a). We examined freshly isolated strains of the pathogen to determine the properties of strains that have not been extensively subcultured. One characteristic associated with many fresh field isolates is "mosaic" morphology (Figure 2). Mosaic cultures derived from a single conidium growing on oatmeal agar are at first smooth and heavily conidiated. Approximately ten days after conidiation, papillae appear in the culture. The papillae can take the form of very heavily conidiated clumps or non-conidiating mycelial outgrowths.

Drops of an extremely viscous ooze also appear on these cultures. The mosaic trait appears to be easily lost, often within a few rounds of subculture on agar plates. When these mosaic strains are subcultured by mass hyphal transfers on oatmeal agar, a progression of morphological changes occurs. The cultures cease to papillate, lose their normal gray pigment, conidiate poorly, and show a phenomenon we describe as "melting out." Melting out appears to involve autolysis of mycelium that is more than 10-14 days old. This degraded condition never reverts to mosaic morphology. Loss of fertility, specifically the loss of the ability to serve as a female in a cross, occurs as rapidly upon subculture as loss of the mosaic morphology. The ability of a strain to serve as a male in a cross appears to be relatively stable. Although pathogenicity is less sensitive to subculturing than female fertility, loss of pathogenicity does occur, and the loss of the ability to produce abundant conidia seriously restricts pathogenicity testing. Thus, it is critical to store all strains in a non-metabolizing state and to recover a strain from storage before any experiment.

Fertility of *Magnaporthe grisea* Field Isolates

We have confirmed reports (Herbert, 1971; Ueyama and Tsuda, 1975; Yaegashi and Nishihara, 1976; Kato et al., 1976; Tanaka et al., 1979; Taga et al., 1982) that field isolates of *M. grisea* exhibit a range of mating characteristics. In our hands, a few field isolates that infect finger millet and goosegrass are hermaphrodites. Many other strains mate only as males and others are totally infertile. Even the most fertile crosses produce only 5-10% viable ascospores. Furthermore, the frequency of ascospore formation and the viability of ascospores are always lower in crosses that involve a rice-pathogenic isolate than in crosses that involve only isolates capable of infecting other grasses. Crosses between any two isolates pathogenic to rice are uniformly infertile, with no perithecia produced, even when the two parents are clearly of opposite mating type, based on their behavior in crosses with non-pathogens of rice. Although Kato and Yamaguchi (1982) have reported crosses between rice pathogens, we have been unable to repeat this result, using the same strains.

Female Sterility Among Rice Pathogens

As shown in Figure 1, a prominent double row of perithecia appears when two hermaphroditic *M. grisea* strains are crossed in the usual manner (see Materials and Methods). We reasoned that the row of perithecia nearest to the site where a particular parent had first been placed on the agar represents mating events in which that

parent has served as the female partner. This supposition received strong support from the following experiment. From strain 281-9-2, we isolated a buff-colored pigment-defective mutant, CP62 (*mat-1 buf-4*), and we mated that mutant with 859-15-4, a wild type strain that produces normal dark gray-colored mycelia. Crosses between two wild type strains produce only dark gray perithecia. When CP62 and 859-15-4 were mated, a distinct double row of perithecia appeared. The row nearest the 859-15-4 parent included hundreds of normal dark gray perithecia, in contrast to the row nearest the CP62 parent, which included hundreds of perithecia with a red color very similar to that of the CP62 mycelium. This result suggests that the tissue of the perithecium is contributed exclusively by one of the parents in any single mating event; we refer to this parent as the female partner.

Whenever one of the parents in a cross is a rice pathogen, only a single row of perithecia is observed. These perithecia are usually positioned such that it appears the non-pathogen of rice has served as the female parent. We have used Buf$^-$ mutants to confirm this observation. Buf$^-$ mutants were produced in the rice pathogens, Ken60-19 (*mat1-2*) and 3862-3 (*mat1-2*). From Ken60-19, we isolated two Buf$^-$ mutants, CP64 (*mat1-2 buf-6*) and CP67 (*mat1-2 buf-9*), and mated these with WGG-FA40, a goosegrass-infecting hermaphrodite. In a typical trial, a mating between CP64 and WGG-FA40 produced about 400 perithecia, all of which showed normal dark gray pigmentation. However, the ascospore progeny of this cross included equal numbers of individuals with Buf$^-$ and Buf$^+$ pigmentation. Similar results were obtained in crosses between CP67 and WGG-FA40, and between CP159 (the *mat1-2 buf-49* strain produced from 3862-3) and the fertile laboratory strain 4090-15-1 (Table 1). These results demonstrate that Ken60-19 and 3862-3 fail to mate as females. The opposite situation occurs when Ken60-19 is crossed with a Buf$^-$ mutant derived from WGG-FA40. In this case all perithecia formed are red. Observation of these and other crosses involving rice pathogens of both mating types (Table 1), as well as our experience in the lab with other rice pathogens, suggests that female sterility is characteristic of most, if not all, rice pathogens. This explains the failure of crosses between any two rice-pathogenic strains of *M. grisea* to produce perithecia.

The SM81-4 Breeding Line

Crosses between two goosegrass-infecting field isolates, SM81-4 and WGG-FA40, yielded asci containing 5 to 10% viable ascospores.

Table 1. Perithecial color as an indicator of hermaphroditism in crosses between rice pathogens and goosegrass pathogens.[a]

Rice-Infecting Parent	×	Goosegrass-Infecting Parent	Perithecial Color
Ken60-19		WGG-FA40 (*buf-26*)	Red
Ken60-19 (*buf-6*)		WGG-FA40	Black
Ina168		4091-5-8 (*buf-57*)	Red
O-42		4091-5-8 (*buf-58*)	Red
3862-3 (*buf-49*)		4090-15-1	Black

[a]Crosses were made between rice pathogens and goosegrass pathogens such that one or the other parent in each cross was marked with a *buf*⁻ mutation. The rice pathogens Ken60-19, Ina168, and O-42 are field isolates, and 3862-3 is a rice pathogen derived from a cross between Ken60-19 and a fertile laboratory strain 3596-5-1. This table lists the *buf*⁻ alleles following the strain in which the *buf*⁻ mutation was produced. The actual strain names are CP64 for Ken60-19 *buf-6*, CP86 for WGG-FA40 *buf-26*, CP159 for 3862-3 *buf-49*, CP281 for 4091-5-8 *buf-57*, and CP282 for 4091-5-8 *buf-58*. For each cross, several hundred perithecia were examined for color; red perithecia are produced by Buf⁻ strains and black perithecia are produced by normally pigmented strains. All perithecia from a particular cross showed the color that would be produced by the goosegrass-infecting parent. This result led us to conclude that the rice-infecting parents mated only as males. Note that the rice pathogens are of both mating types; Ken60-19 and 3862-3 are *mat1-2*, and Ina168 and O-42 are *mat1-1*.

From several independent attempts, a few complete tetrads were isolated in this cross. One mosaic culture derived from a single ascospore progeny from this cross, 70-2-1, appeared to be relatively fertile, as judged by the ability of this strain to produce perithecia when mated with a wide range of other strains. A cross (number 859) between 70-2-1 and the Japanese rice pathogen, O-42, showed 45% germination of ascospores (Figure 3). Seven complete tetrads were isolated from this cross, as well as incomplete tetrad sets. The characteristics of three of these tetrads are shown in Table 2. The ability to mate as a female segregated one-to-one among the progeny of cross 859. The mosaic morphology typical of the 70-2-1 parent also segregated one-to-one. Mosaic morphology and female mating proficiency appeared to be linked (seven parental ditype tetrads were observed). Mating type was not linked to female mating proficiency (six tetrads were tetratypes and the remaining tetrad was a parental ditype).

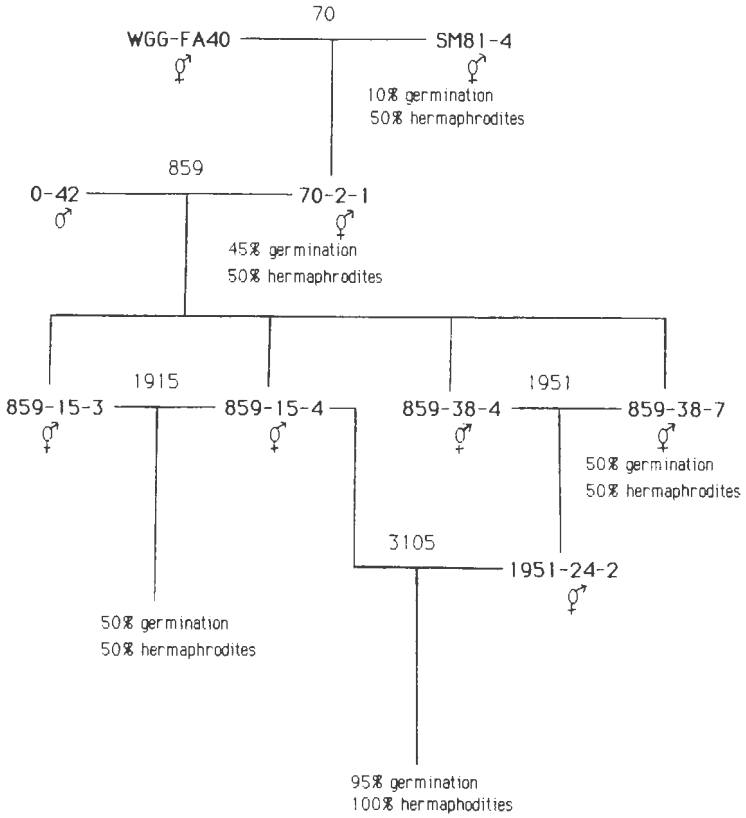


Figure 3. Pedigree chart showing fertility improvement in the SM81-4 breeding line. The field isolates WGG-FA40 and SM81-4, both of which infect goosegrass, undergo sexual crosses in which approximately 10% of the ascospores are viable. Approximately 50% of the progeny are hermaphrodites (♂). A mosaic hermaphroditic strain recovered from this cross, 70-2-1, was crossed with a field isolate that infects rice, 0-42, to give 45% germination of ascospores and 50% hermaphroditic progeny. Progeny from cross 859 were mated, and two crosses were identified, numbered 1915 and 1951, that yielded many more perithecia than cross 859, 50% germination of ascospores, and 50% hermaphrodites. A cross between 859-15-4 and 1951-24-2 is an example of an improved cross in which ascospore viability exceeds 95% and all recovered progeny are hermaphrodites.

Table 2. Analysis of three tetrads from cross 859 between O-42 (a rice pathogen and 70-2-1 (a goosegrass pathogen).[a]

Ascus	Spore	Mating Type	Hermaphrodite	Mosaic Morphology	Pathogenicity to Rice
15	1	*mat1-1*	No	No	Type 0
	2	*mat1-1*	No	No	Type 0
	3	*mat1-1*	Yes	Yes	Type 1-2
	4	*mat1-2*	Yes	Yes	Type 0
	6	*mat1-2*	Yes	Yes	Type 0
	5	*mat1-2*	No	No	Type 0
	8	*mat1-2*	No	No	Type 0
22	1	*mat1-2*	No	No	Type 0
	3	*mat1-2*	No	No	Type 0
	2	*mat1-2*	Yes	Yes	Type 1
	5	*mat1-2*	Yes	Yes	Type 1
	4	*mat1-1*	Yes	Yes	Type 1
	7	*mat1-1*	Yes	Yes	Type 1
	6	*mat1-1*	No	No	Type 0
38	1	*mat1-1*	No	No	Type 1
	5	*mat1-1*	No	No	Type 1
	2	*mat1-2*	No	No	Type 0
	4	*mat1-2*	No	No	Type 0
	3	*mat1-2*	Yes	Yes	Type 0
	6	*mat1-2*	Yes	Yes	Type 0
	7	*mat1-1*	Yes	Yes	Type 0
	8	*mat1-1*	Yes	Yes	Type 0

[a]Progeny from ascus 15, ascus 22, and ascus 38 were scored for mating type, hermaphroditism, morphology, and pathogenicity toward rice. Sister spore pairs were easily identified and are listed together. Mating type, hermaphroditism, and mosaic morphology segregated one-to-one. Hermaphroditism appears to be linked to the mosaic morphology. Mating type is unlinked to these two traits. No rice pathogens were recovered from this cross, although progeny that caused a Type 1 hypersensitive response (unlike either parent) were recovered. See **Materials and Methods** for an explanation of pathogenicity evaluation. A few virulent goosegrass pathogens were recovered among the progeny of this cross.

Progeny pathogenic to rice were not recovered from cross 859 (Table 2). Ninety-six random ascospore cultures were tested in addition to the seven complete tetrads. Some progeny did cause Type 1 hypersensitive resistance reactions on rice cultivar M9, unlike either parent. Virulent pathogens of goosegrass were recovered among the progeny of cross 859.

Selected progeny from cross 859 were mated by one another in all possible combinations in order to determine which crosses would yield the highest percentage of viable ascospores. The germination of ascospores varied in these crosses, with many producing no asci or producing asci containing only nonviable ascospores. Two crosses, 1915 and 1951, shown in Figure 2, produced ascospores with 50% viability. The single ascospore strain, 1951-24-2, was selected as a fertile *mat1-1* hermaphrodite. A cross between this strain and 859-15-4, for example, showed 95% germination of ascospores. All progeny from this cross were hermaphrodites. Both 859-15-4 and 1951-24-2 infect goosegrass. These two strains are examples of fertile laboratory strains derived from less fertile field isolates.

The K76-79 Breeding Line

The weeping lovegrass-infecting field isolate, K76-79, consistently mated with WGG-FA40 to yield ascospores with 45% viability. This is true in spite of the fact that K76-79 had been continuously subcultured on agar slants for several years before we obtained it. K76-79 shows the degraded culture morphology described above, and it is not able to function as a female in crosses. Nevertheless, K76-79 is an aggressive pathogen of weeping lovegrass. Seven complete tetrads from a cross (number 4091) between K76-79 and the goosegrass pathogen, WGG-FA40, have been studied in detail. Three tetrads shown in Table 3 are representative of these seven.

In contrast to the results obtained in cross 859 described above, where 50% of the viable progeny were hermaphrodites, only 4% of the progeny of cross 4091 were hermaphrodites. A limited number of morphological types appeared among the progeny of this cross, many of these exhibiting extremely poor conidiation. Also in contrast to the results in cross 859, determinants of host-species specificity were inherited simply in the K76-79 by WGG-FA40 cross. One half of the progeny within a tetrad were pathogens of weeping lovegrass, and one half were pathogens of goosegrass. Parental and recombinant classes appeared with equal frequency, indicating that a gene determining pathogenicity to weeping lovegrass had segregated independently from a gene determining pathogenicity to goosegrass. Of the seven

Table 3. Analysis of three tetrads from Cross 4091 between the weeping lovegrass pathogen K76-79 and the goosegrass pathogen WGG-FA40.[a]

Ascus	Spore	Mating Type	Hermaphrodite	Pathogenicity to goosegrass[b]	Pathogenicity to weeping lovegrass[b]
5	1	*mat1-1*	No	NT[c]	NT
	2	*mat1-1*	No	NT	NT
	3	*mat1-1*	No	++	−
	4	*mat1-1*	No	++	−
	5	*mat1-2*	No	−	++
	7	*mat1-2*	No	−	++
	6	*mat1-2*	Yes	++	++
	8	*mat1-2*	Yes	++	++
22	1	*mat1-1*	No	++	−
	3	*mat1-1*	No	++	−
	2	*mat1-1*	No	++	−
	4	*mat1-1*	No	++	−
	5	*mat1-2*	No	−	++
	6	*mat1-2*	No	−	++
	7	*mat1-2*	No	−	++
	8	*mat1-2*	No	−	++
25	1	*mat1-1*	No	−	−
	2	*mat1-1*	No	++	++
	4	*mat1-1*	No	++	++
	3	*mat1-2*	No	−	−
	6	*mat1-2*	No	−	−
	5	*mat1-2*	No	++	++
	7	*mat1-2*	No	++	++

[a]Progeny from ascus 5, ascus 22, and ascus 25 were scored for mating type, hermaphroditism, and pathogenicity toward goosegrass and weeping lovegrass. Mating type segregated one-to-one, but hermaphroditic progeny were rare. Pathogenicity toward goosegrass segregated one-to-one, as did pathogenicity toward weeping lovegrass. With regard to goosegrass pathogenicity and weeping lovegrass pathogenicity, ascus 5 is a tetratype tetrad, ascus 22 is a parental ditype tetrad, and ascus 25 is a nonparental ditype tetrad.

[b]+ indicates a pathogen, − indicates a nonpathogen.

[c]Pathogenicity not tested because these strains do not produce conidia.

tetrads examined, two were parental ditypes, one was a nonparental ditype, and four were tetratypes. Thus, the two parental field isolates differ at two unlinked loci, one conditioning pathogenicity toward weeping lovegrass and one conditioning pathogenicity toward goosegrass.

We have performed other crosses to confirm the single gene segregation of host-species specificity. The single ascospore strain from the tetratype tetrad in Table 3, 4091-5-8, a pathogen of both weeping lovegrass and goosegrass, was backcrossed to the goosegrass-infecting parent, WGG-FA40 (cross 4016). Random ascospore analysis was conducted, and 19 of 44 progeny tested for pathogenicity infected only goosegrass, while 25 of 44 infected both goosegrass and weeping lovegrass. The same strain, 4091-5-8, which infects both goosegrass and weeping lovegrass, was crossed with 4091-14-1, a strain that infects only weeping lovegrass and has better morphological characteristics than the parent K76-79 (cross 4127). From this cross, whole tetrads were readily recovered. These tetrads included only two types of pathogens, those that infected only weeping lovegrass and those that infected both weeping lovegrass and goosegrass. These crosses thus support the conclusion of single gene segregation of host-species specificity differences between the Japanese field isolates, WGG-FA40 and K76-79.

In the K76-79 breeding line, the selection and subsequent crossing of hermaphroditic progeny has quickly resulted in improved fertility. For example, in such a cross (number 4127), one between 4091-5-8, which infects both weeping lovegrass and goosegrass, and 4091-14-1, which infects weeping lovegrass, more than 90% of the ascospores germinate. Approximately 50% of the progeny of this cross are hermaphrodites. The parents in this cross (number 4127) were selected partly because they showed mosaic morphology. This facilitated genetic analysis of pathogenicity, as the progeny from a cross between these two improved strains conidiated better and were therefore easier to test for pathogenicity.

The Ken60 Breeding Line

The failure to recover rice pathogens from crosses in the SM81-4 breeding line prompted investigation of crosses between the rice pathogen, Ken60-19, and the goosegrass pathogen, WGG-FA40. Twentyfive percent of the progeny of a cross between these two strains were reported to be pathogens of rice (Yaegashi and Asaga, 1981). In our experience, the results of a cross between WGG-FA40 and Ken60-19 are variable, and it must be repeated in order to obtain

successful mating. Ascospore germination is consistently rare; we have never observed more than 10% viability. Relatively few asci are produced; many of them contain seven or eight ascospores with normal appearance but poor viability. Most tetrads do not contain any viable ascospores, a few contain two viable ascospores, and even fewer contain four viable ascospores. Examination of the surviving ascospores suggests they are sister spore pairs, mitotically derived from a single product of the second meiotic division; i.e., the live spores isolated from an ascus routinely can be grouped in pairs with the same morphological characteristics, the same mating type and fertility, and the same pathogenicity.

Progeny from the WGG-FA40 by Ken60-19 cross were obtained either by tetrad dissection or by isolating intact asci and retaining those with ascospores that germinated. Special care was taken to ensure that progeny cultures were not derived from conidia but had indeed arisen from an ascus or an ascospore. When intact asci were used, cultures were considered as impure. Thus, any progeny culture that was recovered from an ascus was purified by isolating a single conidium before morphological, mating, and pathogenicity properties were assigned. Approximately 40% of the progeny produced enough conidia to be tested for pathogenicity. Among these, 20% were vigorous pathogens of rice (Type 5 infectors). Approximately 13% of the tested progeny showed pathogenicity toward goosegrass. Equal numbers of progeny showed *mat1-1* and *mat1-2* mating types. One percent of the progeny were hermaphrodites. While hermaphrodites and goosegrass pathogens exhibited the two mating types with equal frequency, the Type 5 rice pathogens were consistently *mat1-2*, the same mating type as Ken60-19. Type 3 rice pathogens (3% of the progeny tested) were all *mat1-1*, the opposite mating type from Ken60-19.

In an effort to enhance their fertility, rice pathogens obtained from the WGG-FA40 by Ken60-19 crosses were backcrossed to WGG-FA40. Rice pathogens recovered from these backcrosses were again mated with WGG-FA40. Two separate lines of crosses have been followed in this manner for four generations (one of these is shown in Figure 4). Although many rice pathogens have been recovered with fertility equal to or less than that of Ken60-19, no rice pathogens derived from this breeding scheme appear more fertile than Ken60-19. Crosses between fourth generation rice pathogens and WGG-FA40 still show the same pattern of ascospore lethality observed in the WGG-FA40 by Ken60-19 cross.

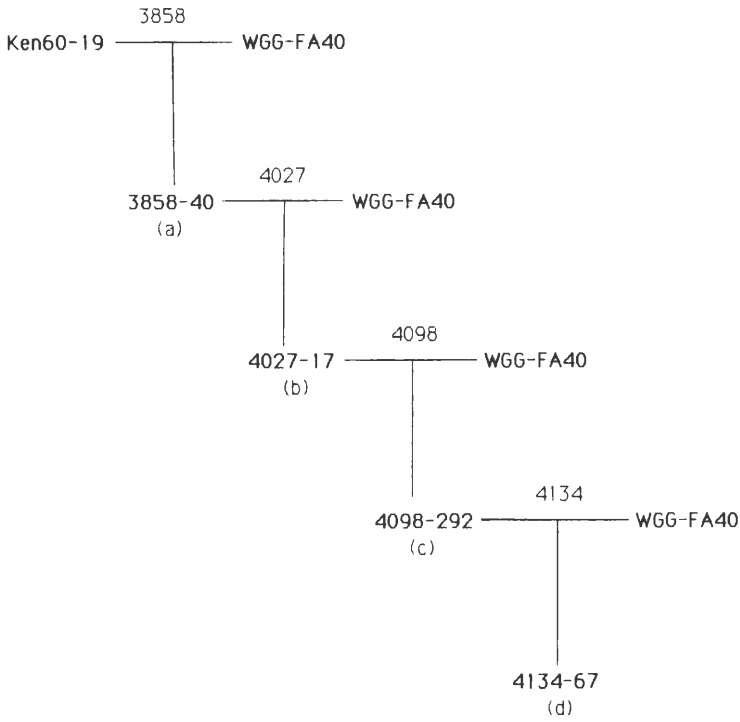


Figure 4. One backcrossing program in an attempt to create hermaphroditic rice pathogens. Designates are as follows: a) one of the nine rice pathogens recovered from 49 tested progeny of the cross 3858; b) the only rice pathogen recovered from eight tested progeny of cross 4027; c) one of 15 rice pathogens recovered from 82 tested progeny of cross 4098; d) one of two rice pathogens recovered from 35 tested progeny of cross 4134. The nine rice pathogens recovered from cross 3858 between Ken60-19 and WGG-FA40 did not show improved fertility compared to Ken60-19. One of these, 3858-40, was backcrossed to the hermaphroditic parent, WGG-FA40, in a further attempt to obtain a rice pathogen with improved fertility. This process was repeated two more times. None of the 27 rice pathogens obtained in these crosses showed improved fertility when compared to Ken60-19. Some did show decreased fertility.

Thirty rice pathogenic progeny derived from the crosses just described were mated with sibling hermaphrodites that are non-pathogens of rice. Ken60-19 was also crossed with the same hermaphrodites. The hermaphroditic progeny derived from the Ken60-19 breeding line cross with each other and with WGG-FA40 to yield numerous ascospores with greater than 90% germination. However, crosses between these hermaphrodites and any of the rice pathogens were similar to or worse than the original cross between Ken60-19 and WGG-FA40. Strain 4098-250 is a *mat1-1* hermaphrodite derived from the Ken60-19 breeding line that produced 100 times more perithecia than WGG-FA40 in crosses with its sibling rice pathogens. Strain 4098-250 was able to form perithecia with all rice pathogens derived from the Ken60-19 breeding line, even though one third of these strains never formed perithecia with WGG-FA40 or any other strain. However, these crosses never resulted in the formation of asci. This is one of several examples of a separation between the ability to produce perithecia and the ability to produce viable ascospores.

DISCUSSION

Natural isolates of *Magnaporthe grisea* differ in host range, both with regard to the ability to attack various species of grasses and with regard to the ability to infect different cultivars of rice. The poor fertility of field isolates of the fungus presents a significant obstacle to conducting a satisfactory genetic analysis of these host range differences. The results presented in this paper demonstrate that it is possible to develop *M. grisea* laboratory strains that show substantial improvements over field isolates in features of fertility, such as production of perithecia and viable ascospores, or features of morphology in culture, such as abundant production of conidia. The success of breeding programs designed to develop fertile pathogenic laboratory strains is primarily determined by the inheritance pattern of differences in fertility and pathogenicity. Depending on the *M. grisea* strains that were crossed and the aspects of fertility or pathogenicity under consideration, we have seen one-to-one segregation ratios that suggest single gene differences, and we have seen complex segregation ratios that suggest polygenic differences.

Although there has been a report of a successful mating between *M. grisea* rice pathogens (Kato and Yamaguchi, 1982), we have been unable to reproduce that result, using the same strains. Furthermore, we have been unable to cross any two rice-pathogenic strains in our collection. Results presented in this paper confirm reports (Tanaka

et al., 1979; Yaegashi and Asaga, 1981) that rice pathogens will mate successfully with hermaphroditic pathogens of other grasses, yielding viable ascospores. Thus, this work supports the idea that there should be no species distinction between strains of the pathogen that infect rice and those that infect other grasses. Results presented in this paper confirm one other report that, regardless of mating type, rice pathogens are only able to serve as males in sexual crosses (Itoi et al., 1983). This is true of the *mat1-1* field isolates, O-42 and Ina168, and the *mat1-2* field isolate, Ken60-19. Quite different results were obtained when the rice pathogens, O-42 and Ken60-19, were mated in separate crosses with a hermaphroditic pathogen of goosegrass. One-half of the progeny of a cross between O-42 and 70-2-1 (Cross 859) were hermaphrodites, suggesting that O-42 carries just a single gene defect in perithecial formation. Only 1% of the progeny of crosses between Ken60-19 and WGG-FA40 were hermaphrodites, however, suggesting that Ken60-19 may lack a number of genes required for formation of perithecia. From both crosses, hermaphrodites among the progeny showed each mating type with equal frequency. Efforts to derive a hermaphroditic rice pathogen were thwarted in both crosses: the cross with O-42 yielded no rice pathogens, while the cross with Ken60-19 yielded 20% rice pathogens, none of which were hermaphrodites. We do not, however, regard these data as conclusive evidence that hermaphroditism and rice pathogenicity are mutually exclusive characteristics in *M. grisea*. We will persist in efforts to develop fertile rice pathogens, turning our attention to other field isolates from other geographical areas.

Crosses reported herein provide insight into the genetic differences between field isolates of *M. grisea* that account for host species specificity. Cross 859 between the rice-infecting field isolate O-42 and the goosegrass-infecting strain 70-2-1 gave no rice pathogens among 150 progeny tested. This suggests (1) that a number of genes act together to determine the ability to infect rice and (2) that some non-pathogens of rice from the field lack several of these genes. Crosses between the rice pathogen, Ken60-19, and the goosegrass pathogen, WGG-FA40, suggest that there are fewer gene differences between these strains that are critical for infecting rice, because about 20% of the testable progeny were rice pathogens. Similarly, Ken60-19 and WGG-FA40 differ in several genes that play a role in pathogenicity on goosegrass, because 13% of the progeny from this cross were goosegrass pathogens, like WGG-FA40. In contrast, the weeping lovegrass pathogen, K76-79, which does not infect goosegrass, mates with WGG-FA40 to yield many viable progeny, half of which can infect

goosegrass. This indicates that a single gene difference between K76-79 and WGG-FA40 accounts for the pathogenicity difference on goosegrass. Furthermore, the same cross shows that alleles of another, unlinked gene determine pathogenicity toward weeping lovegrass.

Single gene segregation of determinants of host-species specificity has previously been reported in *M. grisea*. Yaegashi (1978) crossed a field isolate that infects weeping lovegrass but not finger millet with three different field isolates that infect finger millet but not weeping lovegrass. In all three crosses, the ability to infect weeping lovegrass segregated one-to-one among the progeny, as did the ability to infect finger millet. Most of the progeny resembled one or the other of the parents in being restricted either to finger millet or to weeping lovegrass, although some could infect both hosts and others could infect neither. This indicates that the weeping lovegrass pathogen differs from the three finger millet pathogens at two linked loci, one of which determines weeping lovegrass pathogenicity, while the other determines finger millet pathogenicity. The gene(s) for weeping lovegrass pathogenicity that Yaegashi thus identified may or may not be the same as the weeping lovegrass pathogenicity gene revealed by the crosses described in this paper. Although the weed, goosegrass (*Eleusine indica*) is a close relative of the grain crop, finger millet (*Eleusine coracana*), it seems unlikely that Yaegashi's determinant of finger millet pathogenicity is the same gene as ours of goosegrass pathogenicity; i.e., on our results that clearly show no linkage to a gene that governs pathogenicity toward weeping lovegrass.

In the early days of genetic studies with *Neurospora crassa*, Lindegren et al. (1939) observed the spontaneous appearance of mutants with defects such as "peach" pigmentation or "clump" morphology in strains that had been maintained by serial transfer on agar slants. Many properties of *M. grisea* strains change during continuous subculture; some of these changes could be due to mutation. Problems with loss of fertility and pathogenicity can clearly be overcome by storing the fungus in a non-metabolizing state, as we have described.

Some of the results reported in this paper conflict with earlier published studies. We cannot repeat Kato and Yamaguchi's crosses (1982) between two *M. grisea* rice pathogens; in our hands, the strains are even unable to infect the M9 cultivar of rice, which has no known resistance to blast disease. These incongruities may be a consequence of the maintenance of the relevant *M. grisea* strains by serial transfers before we received them.

Results presented in this paper have identified single genes that govern the ability to attack different species of host grasses. Our goal now is to continue to improve genetic analysis in *M. grisea*, to identify genes that determine cultivar specificity among the rice pathogens, to identify genes that determine general pathogenicity, and to identify additional genes that determine host species specificity. The identification of critical genes will lead to efforts to understand how these genes act in determining the outcome of a particular host pathogen interaction. A first step would be to determine whether the pathogenic or non-pathogenic allele is dominant at each of these genes (Crawford et al., in press). This knowledge will provide an indication of whether these genes act to permit infection by *M. grisea*, like the host specificity determinant described in *Nectria haematococca* (Tegtmeier and VanEtten, 1982), or whether these genes act to exclude *M. grisea* from certain hosts, like classical avirulence genes (Day, 1974). A detailed understanding of general pathogenicity and host specificity of *M. grisea* will lead to a detailed understanding of resistance mechanisms in host plants. We believe that this understanding will lead to insights for controlling plant diseases, perhaps by aiding in the design of stable disease resistant cultivars of crop plants.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of the Department of Energy (DE-ACO2-76ERO-1426 and DE-ACO2-84ER13160), which allowed us to initiate this work. We are also grateful for support from The Rockefeller Foundation (RF81042), and from Monsanto Agricultural Products Company. We would like to acknowledge the support and encouragement given by Dr. Peter Albersheim in whose laboratory this work was performed. We would like to thank Dr. Frances Latterell for introducing us to *Pyricularia* and for supplying fungal cultures. Many techniques currently in use in our laboratory were developed by Dr. Latterell. We are especially grateful to Dr. Hajime Kato and Dr. Hiroshi Yaegashi for sharing strains of *Pyricularia*, and for providing helpful insights on the *Pyricularia*. Dr. Kato generously arranged field trips for BV in Ibaraki and Tochigi Prefectures in Japan in July, 1982, for the purpose of collecting fresh field isolates of the fungus. Dr. Yaegashi has generously collected fresh field isolates of the pathogen at our request. We would also like to thank Kenneth A. Parsons for assistance with pathogenicity assays included in this work.

LITERATURE CITED

Asuyama, H. 1965. Morphology, taxonomy, host range, and life cycle of *Piricularia oryzae*, pp. 9-22. *In*: The rice blast disease. Proceedings of a Symposium at the International Rice Research Institute, July, 1963. The Johns Hopkins Press, Baltimore.

Atkins, J. G. and collaborators. 1967. An international set of rice varieties for differentiating races of *Piricularia oryzae*. Phytopathology 57:297-301.

Barr, M. E. 1977. *Magnaporthe*, *Telimenella* and *Hyponectria* (Physosporellaceae). Mycologia 69:952-966.

Crawford, M. S., F. G. Chumley, C. G. Weaver, and B. Valent. In press. Characterization of the heterokaryotic and vegetative diploid phases of *Magnaporthe grisea*. Genetics.

Day, P. R. 1974. Genetics of host-parasite interaction. W. H. Freeman, San Francisco.

Hebert, T. T. 1971. The perfect stage of *Pyricularia grisea*. Phytopathology 61:83-87.

Itoi, S., T. Mishima, S. Arase, and M. Nozu. 1983. Mating behavior of Japanese isolates of *Pyricularia oryzae*. Phytopathology 73:155-158.

Kato, H. 1978. Biological and genetic aspects in the perfect state of rice blast fungus *Pyricularia oryzae* Cav. and its allies, pp. 1-22. *In*: Gamma field symposia No. 17. Mutation breeding for disease resistance.

———— and T. Yamaguchi. 1982. The perfect state of *Pyricularia oryzae* Cav. from rice plants in culture. Ann. Phytopath. Soc. Japan 48:607-612.

————, ————, and N. Nishihara. 1976. The perfect state of *Pyricularia oryzae* Cav. in culture. Ann. Phytopath. Soc. Japan 42:507-510.

Kiyosawa, S. 1976. Pathogenic variations of *Pyricularia oryzae* and their use in genetic and breeding studies SABRAO J. 8:53-67.

Latterell, F. M., M. A. Marchetti, and B. R. Grove. 1965. Coordination of effort to establish an international system for race identification in *Pyricularia oryzae*, pp. 257-274. *In*: The rice blast disease. Proceedings of a Symposium at the International Rice Research Institute, July, 1963. Johns Hopkins Press, Baltimore.

Leung, H. 1984. Genetic and cytological characterization of the rice blast fungus, *Pyricularia oryzae* Cavara. Ph.D. Thesis. Univ. Wisconsin, Madison.

Lindegren, C. C., V. Beanfield, and R. Barber. 1939. Increasing the fertility of *Neurospora* by selective inbreeding. Botanical Gaz. 100:592-599.

Nagakubo, T., M. Taga, M. Tsuda, and A. Ueyama. 1983a. Sexuality in *Pyricularia oryzae*. Mem. Coll. Agric., Kyoto Univ. 122:53-59.

————, ————, ————, and ————. 1983b. Genetic studies on auxotrophic characteristics of *Pyricularia oryzae*. Mem. Coll. Agric., Kyoto Univ. 122:67-73.

———, ———, ———, and ———. 1983c. Genetic linkage relationships in *Pyricularia oryzae*. Mem. Coll. Agric., Kyoto Univ. 122:75-83.

Ou, S. H. 1980. Pathogen variability and host resistance in rice blast disease. Ann. Rev. Phytopathol. 18:167-187.

Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can. J. of Microbiology 8:591-594.

Taga, M., H. Nakagawa, M. Tsuda, and A. Ueyama. 1978. Ascospore analysis of Kasugamycin resistance in the perfect stage of *Pyricularia oryzae*. Phytopathology 68:815-817.

———, ———, ———, and ———. 1979. Identification of three different loci controlling Kasugamysin resistance in *Pyricularia oryzae*. Phytopathology 69:463-466.

———, T. Waki, M. Tsuda, and A. Ueyama. 1982. Fungicide sensitivity and genetics of IBP-resistant mutants of *Pyricularia oryzae*. Phytopathology 72:905-908.

Tanaka, Y., N. Murata, and H. Kato. 1979. Behavior of nuclei and chromosomes during ascus development on the mating between either rice-strain or weeping lovegrass-strain and ragi-strain of *Pyricularia*. Ann. Phytopath. Soc. Japan 45:182-191.

Tegtmeier, K. J. and H. D. VanEtten. 1982. The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: A genetic analysis. Phytopathology 72:608-612.

Ueyama, A. and M. Tsuda. 1975. Formation of the perfect state in culture of *Pyricularia* sp. from some graminaceous plants (preliminary report). Trans. Mycol. Soc. Japan 16:420-422.

Vogel, H. J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Amer. Nat. 98:435-446.

Waki, T., M. Taga, M. Tsuda, and A. Ueyama. 1983. Utilization of auxotrophic mutants for ascospore analysis of *Pyricularia oryzae*. Mem. Coll. Agric., Kyoto Univ. 122:61-66.

Yaegashi, H. 1978. Inheritance of pathogenicity in crosses of *Pyricularia* isolates from weeping lovegrass and finger millet. Ann. Phytopath. Soc. Japan 44:626-632.

——— and K. Asaga. 1981. Further studies on the inheritance of pathogenicity in crosses of *Pyricularia oryzae* with *Pyricularia* sp. from finger millet. Ann. Phytopath. Soc. Japan 47:677-679.

——— and T. T. Hebert. 1976a. Perithecial development and nuclear behavior in *Pyricularia*. Phytopathology 66:122-126.

——— and ———. 1976b. Effect of temperature, light, and nutrients on production of perithecia by *Pyricularia*. Ann. Phytopath. Soc. Japan 42:556-562.

———— and N. Nishihara. 1976. Production of the perfect stage in *Pyricularia* from cereals and grasses. Ann. Phytopath. Soc. Japan 42:511-515.

———— and S. Udagawa. 1978a. The taxonomical identity of the perfect state of *Pyricularia grisea* and its allies. Can. J. Bot. 56:180-183.

———— and ————. 1978b. Additional note: the perfect state of *Pyricularia grisea* and its allies. Can. J. Bot. 56:2184.

Yamada, M. and collaborators. 1976. Proposal of a new method for differentiating races of *Pyricularia oryzae* Cavara in Japan. Ann. Phytopath. Soc. Japan 42:216-219.